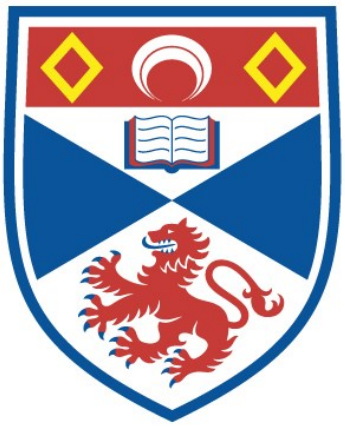
CALCIUM AND AGEING IN THE ROTIFER MYTILINA BREVISPINA VAR REDUNCA

A. M. Sincock

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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CALCIUM AND AGEING

IN THE ROTIFER MYTILINA BREVISPINA VAR REDUNCA

BY

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DEPARTMENT OF NATURAL HISTORY

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PART I

INTRODUCTION

The rotifera are acoelomate triploblastic animals that are found in most aquatic or moist habitats. The remarkable power of resisting dessication in the eggs and adults of some species permits the exploitation of ephemeral bodies of water. Some species are parasitic but most are free-living and of cosmopolitan distribution with specific preferences as to pH, salinity and gas content of the water. Their most characteristic external feature is a unique arrangement of cilia at the oral end, that is sometimes referred to as a Wheel Organ, and plays an important part in the processes of feeding and sometimes locomotion. A considerable morphological range is to be found in the phylum though the following features are common to females of all species, an outer partially or fully developed cuticle or lorica to which are attached muscles and connectives that traverse a syncitial body mass, a complex alimentary canal ciliated at its oral opening and opening to the exterior at its posterior extremity - the anus, a simple nervous system composed often of only a single ganglion and a small number of longitudinal nerves, a simple nephridial exretory system terminating in a bladder that opens to the exterior, and a single or paired ovary composed of germarium and germovitellarium. The entire phylum is divisble into two principle Classes, the Digononta that possess two ovaries, a high degree of body contractility and always produce females that reproduce by ameiotic diploid parthenogenesis, and the Monogononta that possess only one ovary, cannot contract parts of the body, and in addition to producing females that reproduce parthenogenetically may sometimes produce morphologically simplified individuals (males) that may take part in a process of sexual reproduction termed "mixis". The rotifer chosen for this present study belonged to the Class Monogononta and more specifically to the Order Ploima, Family Salpinidae, and genus Mytilina.

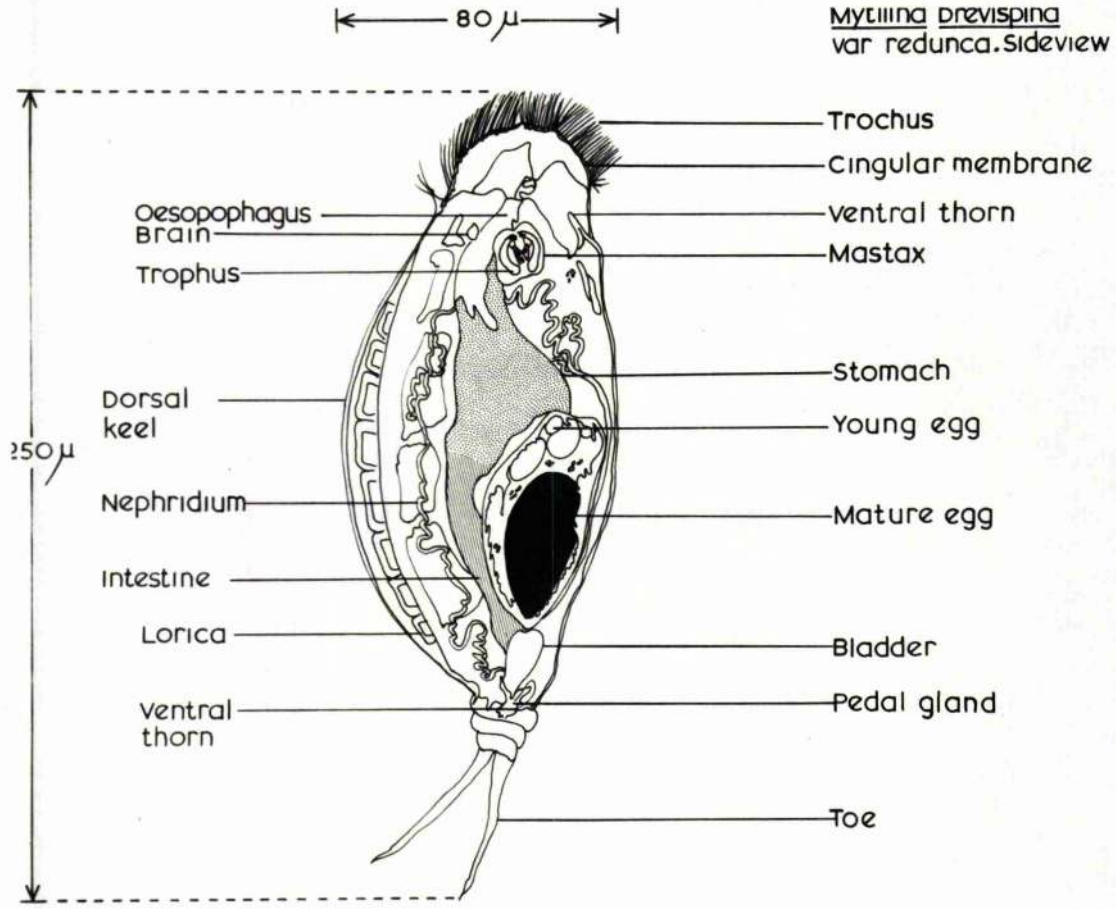

80 µ
250 µ
Mytilina brevispina
var redunca. Sideview
Trochus
Cingular membrane
Ventral thorn
Mastax
Stomach
Young egg
Mature egg
Bladder
Pedal gland
Toe
Oesopophagus
Brain
Trophus
Dorsal keel
Nephridium
Intestine
Lorica
Ventral thorn

Diagram 1

The Monogonont order Ploima contains rotifers that possess feet, are fully or partially loricate, and are free-living or parasitic. The Family Salpinidae belongs to the fully loricate group of the Order Ploima and is characterised by two modifications of the lorica, one in the mid dorsal line forming a pronounced ridge or keel, and the other at the anterior and posterior ventral extremities forming characteristic processes or 'Thorns'. In the genus Mytilina with the exception of one species Mytilina petryi, the mid dorsal keel is well-developed extending over almost the entire length of the dorsal surface, and in the particular species adopted for this present study Mytilina brevispina var redunca, the ventral processes or thorns are reflected back towards the dorsal line. This feature together with the complete absence of males and sexual reproduction (mixis), from any part of the life-cycle constitutes the principle differences between Mytilina brevispina var redunca and the more commonly found Mytilina brevispina.

(A diagram of Mytilina brevispina var redunca is shown opposite.)

Mytilina brevispina var redunca constitutes a suitable organism for ageing studies since it reproduces only by ameiotic diploid parthenogenesis thereby producing no genetic variation (excluding mutations) in its offspring, and characteristically of its Class the Monogononta, it possesses a conveniently short life-span. In common with rotifers generally, it features a determinate and eutelic embryological development, such that variations in adult cell age and number did not occur between individuals. Finally, the species could be cultured on simple and standard culture media, yet still maintain a reproductive level that enabled large numbers to be bred for experiments in a relatively short time.

Representative species of both Classes of rotifers the Digononta and Monogononta have been used in the past by different authors in Ageing studies. Most of the Digononts selected for such work belonged to a principle family of the Bdelloidea, the Philodinidae, while most of the Monogononts were selected from a range of families such as the Euchlanidae, the Notommatidae, and the Brachionidae, all families

of the largest Order of the Class, the Order Ploima. A Table has been drawn up summarising the approximate range of rotifer species adopted in ageing studies within the last 60 years. Alongside each species is a summarised title of the work (not necessarily the original title) and the date it was published together with the author(s) who performed it. It should be stressed that the Table does not claim to be a complete record of every rotifer species employed in every ageing study to date.

TABLE 1

SPECIES	SUMMARISED TITLE OF WORK PERFORMED	DATE	AUTHOR(S)
DIGONONTA			
Philodina citrina	Orthoclone studies on the transmissability and reversibility of an ageing factor that is related to an increase in cellular Ca.	1947	Lansing A.I.
Philodina citrina	Further orthoclone studies linking onset of Ageing with cessation of Growth.	1948	Lansing A.I.
Philodina citrina	Further orthoclone studies on a non genic ageing factor.	1954	Lansing A.I.
Philodina megalotrocha	Effect of pH, Total Salt Concn on the longevity value of this rotifer.	1959	Pray F.A.
Philodina acuticornis	Effect of underfeeding and temperature changes on longevity, and biochemical characteristics at different ages.	1965	Fenestil D.D. Barrows C.H.
Philodina acuticornis	Effects of various culture systems on longevity, fecundity and biochemical characteristics at different ages.	1969	Meadow N.D. Barrows C.H.
Philodina acuticornis	Age related changes in the ultrastructure and histo-chemistry of rotifer organs.	1970	Meadow N.D. Herold R.C.
Rotifer vulgaris	Effect of calcium on longevity value.	1942	Lansing A.I.

MONOGONONTA

Proales sordida	Effect of food level on longevity with special reference to heritability of such effects.	1931	Lynch R.S. Smith H.B.
Proales sordida	Age, Mortality, Fertility and individual diversities in the rotifer Proales sordida. 1. Effect of parental age on offspring.	1928	Lynch R.S. Jennings H.S.
Proales sordida	Age, Mortality, Fertility and individual diversities in the rotifer Proales sordida. 2. Life history in relation to mortality and fecundity.	1928	Lynch R.S. Jennings H.S.
Proales sp.	Effect of citrate treatment and total salt concn on longevity of this rotifer.	1942a	Lansing A.I.
Proales decipiens	Experimental studies on life history.	1922	Noyes B.
Hydatina senta	Comparison of longevity in amictic and mictic forms of this species.	1932	Ferris J.C.
Hydatina senta	Age and vigour in relation to cross fertilization and varied environmental regimes.	1912a,b	Whitney D.D.
Distyla inermis	Alternation of Generations and death by reproductive exhaustion hypothesis.	1931	Miller H.M.
Distyla inermis	Effect of physical and chemical agents on fecundity longevity and the heritability of such effects.	1926	Finesinger J.
Brachionus pala	Reproduction, growth, longevity and inheritence in this species.	1934	Chu J-P.
Brachionus sp.	Relationships between food value and mean longevity.	1960	Pourriot R.
Euchlanis triquetra	Effect of pH on longevity value.	1942a	Lansing A.I.
Euchlanis dilatata	Increase in cortical calcium with age as shown by micro-incineration technique.	1942b	Lansing A.I.
Euchlanis dilatata	Food, Age and Dynamics in a rotifer population.	1967	King C.E.
Euchlanis triquetra	Diversity in egg measurements as a function of age in this species. (In Review Article).	1969	King C.E.

Several recent studies (see Table 1) have employed the Digonont rotifer Philodina acuticornis odiosa (Fanestil and Barrows 1965, Meadow and Barrows 1969, and Herold and Meadow 1970). The mean life-span of this rotifer cultured on a dixenic bacterial culture regime based on dried grass infusion was 27 days (Meadow and Barrows 1969), with a maximum egg production of 7 to 8 eggs per day. When lactate and malate dehydrogenase activities were measured in homogenates of single rotifers as a function of dry weight and age, these authors found that decrements occurred in the activities of both these enzymes that were related to increased age and increased protein content. It was also suggested by Meadow and Barrows 1969 that marked age-related changes took place in the synthesis of specific proteins (the concentration of R.N.A. was found to increase with age) and this was in agreement with the study of Herold and Meadow 1970 performed on the same rotifer, that found age-related increments in the amounts of acid phosphatase associated with inclusion bodies. Both authors of the 1969 paper postulated that increases in lysosomal enzymes could account for increases in protein in old rotifers and these enzymes could bring about cell death by autolysis. Part of the protein increase could be due, they suggested, to an increase in the production of non-functional protein capable of impairing cellular metobolism.

Both of the studies performed on Philodina acuticornis by Fanestil and Barrows 1965 and Meadow and Barrows 1969, threw new light on the effects of artificial culture regimes on rotifers. The 1969 study was the first to employ fully aseptic culture conditions, this being deemed necessary by the discovery that bacterial contamination of cultures produced longevity variation in rotifers (possibly by providing an additional and uncontrolled food source King 1969, Meadow and Barrows 1969). Temperature fluctuations of the order of $1^{o}C$ could produce over the course of a single life-span in this rotifer variations in longevity value of up to 3 days (Meadow unpublished data) a discovery that prompted the strictest possible temperature control of cultures.

The Bdelloid rotifer Philodina citrina has been the subject of an interesting series of cloning experiments performed by Lansing in 1947, 1948 and 1954. Lansing observed generations of isogenic clones from this organism that were termed "orthoclones" because they were rendered uniform with respect to maternal age by the repeated selection of progeny from mothers of a given age over the course of generations. Lansing compared the longevities of individuals that were the progeny of repeated early born selections with those that were the progeny of repeated late-born selections. He showed a conclusive reduction in longevity for the late-born selected progeny that could be reversed by switching to early born selection, and it was results such as these that led him to conclude in 1947 that parental age effects the life-span of progeny. Further, as there was an inverse relationship between the age of an orthoclone and the number of generations required to bring about its extinction, Lansing postulated in his 1947, 1948 and 1954 papers that in the absence of genetic variation (Philodina was an obligate parthenogen) there existed in his orthoclones a cytoplasmic factor that was both cumulative and transmissable between generations. In his 1947 paper Lansing referred to orthoclones that displayed decreasing life-spans over generations, as "geriaclones", while orthoclones that exhibited increasing life-spans over generations were termed "pediaclones". The point in life at which a pediaclone became a geriaclone in the case of Philodina citrina was found by Lansing in 1948 to coincide with the exact time of growth cessation, i.e. between days 5 and 6. This experimental result related to Lansings 1942b study on the Monogonont rotifer Euchlanis dilatata, where it was found that calcium (identified by the sodium alizarin sulphonate reaction) did not appear in microincinerated tissues of this organism until after growth had been completed. These results in conjunction with the results of his 1942a paper, where it was shown that the life-span of the rotifer Rotifer vulgaris increased when this species was cultured on a modified Knop's medium that featured a low calcium - high potassium balance, and that of the rotifer Proales sp. increased when individuals were subjected to brief and irregular immersions in 0.5% sodium citrate (that allegedly removed calcium) throughout life, led Lansing to postulate that the ageing factor in his orthoclones together with the ageing factor in unselected individuals could be related to increases in calcium content during ageing.

Much of the early work performed on Monogonont rotifers is difficult to assess on the grounds that non standard techniques were employed in their culture. The study of Lynch and Smith in 1931 on the effects of food levels and their inheritance in the rotifer Proales sordida, while it showed that there was a depreciation in longevity and fecundity in rotifers reared on quarter strength and half strength oatmeal medium compared to the full strength controls (strengths being assessed by the number of flakes employed per 100 ccs of spring water) also showed gross temperature variations in certain phases of the work, totally undefined culture media, and an absence of aseptic or even partially aseptic culture procedures. The study of Miller 1931, performed on the Monogonont rotifer Distyla inermis, which compared egg-laying and longevity in mictic and amictic females of this species, and led to a theory of Ageing based on reproductive exhaustion, again becomes very difficult to assess on the grounds of undefined and non standard culture procedures.

More recently studies such as those of Pourriot on members of the Monogonont family Brachionidae have contributed a great deal to a knowledge of the effects of different nutritional species on longevity and fecundity. Pourriot showed in 1960 that there was no clear relationship between the level of nutritional regime and the mean longevity of several species of Monogonont rotifers, this being the reverse of the findings of Fanestil and Barrows 1965 considered earlier in this text.

The experiments of Lansing performed on Proales sp. in 1942 have already been superficially considered. They involved briefly immersing this rotifer in a 0.5% solution of sodium citrate for approximately one minute on days 2,4,6,7,8 and 9 after hatching. Individuals were then returned to their droplets of Knops Control medium, where they apparently showed no ill effects as a result of their treatment. The mean longevity value of 30 citrate treated control rotifers was 8.3 ± 0.5 days compared with 5.8 ± 0.5 days for the same number of untreated controls. Quite apart from the longevity increase in the citrate treated group which Lansing attributed to the specific removal of calcium from the rotifers by

sodium citrate, there was also a greatly increased egg production, with egg-laying continuing in the extended parts of the life-span. Lansing attributed great significance to the level of egg-laying in experimental populations that displayed increased life expenctancy. Previously Finesinger in 1926 had shown that mean length of life and temperature were inversly related, a result that was confirmed by Fanestil and Barrows in 1965. This relationship was explained by Finesinger and Lansing on the basis of an increased metabolic rate at higher temperatures that resulted in an acceleration of certain phases of the life cycle. It was this sort of relationship between a physical parameter and metabolic rate that led Lansing in 1942 to state that in order to analyse the mechanism of ageing per se and the factors controlling it, it is necessary that experimental procedures be employed that directly influence the ageing process without retarding metabolic activity during the other phases of the life cylce. On the basis of his assumption that egg production could be taken as an index of the level of metabolism Lansing considered that the increased life expectancy (which was associated with increased egg laying in the sodium citrate treated population), occurred as a result of the retardation of the ageing processes per se and not as a result of a reduced metabolic status. The level of egg-laying has been taken in many ageing studies to date as reflecting the level of metabolism, Finesinger 1926, Lansing 1942, Fanestil and Barrows 1965 etc., although no metabolic measurements have to my knowledge been carried out to verify this assumption. I would thus suggest that such an assumption be adopted with great caution although in the absence of any direct measurement of metabolic rate it does seem likely that an intrinsic process such as egg laying could be tentatively considered as an index of gross physiological changes induced by experimental procedures. It is interesting to note that in his 1942 experiments on the effects of total salt concentration on mean longevity in the rotifer Proales sp, a 0.04% Knop's medium produced a greater mean longevity than did a 0.02% Knop's medium. This longevity increase occurred at the expense of reduced egg-laying and hence on the basis of Lansing's reasoning outlined above was not necessarily a retardation of the ageing processes per se, but may have arisen from a reduction in metabolic rate. It is

interesting to note that the reverse result was obtained in the same experiment for the Bdelloid rotifer Rotifer vulgaris, namely at 0.02% total salt concentration mean longevity in Knop's medium was increased, and this increased life-span was not accompanied by a reduction in egg-laying. The difference between the two results for Proales sp and Rotifer vulgaris emphasises the effect of species differences in rotifers, and this is particularly relevant to the first part of this work where much care was devoted to establishing culture regimes that fitted the species requirements of the rotifer chosen for this present study, namely Mytilina brevispina var reduncа.

Despite the range of rotifer species employed in ageing studies to date very little is known concerning the requirements of different species in either laboratory or artificial habitats. Undoubtedly a major obstacle in this respect has been the lack of aseptic and standardised culture techniques in laboratory culture procedures. However, the work of Pourriot on the effects of nutrient species and fecundity, the work of Dougherty on the synxenic and axenic culture of several species of rotifers, and more recently the work of Meadow and Barrows on the effects of dixenic aseptic culture procedures on longevity, fecundity, and biochemical characteristics at different ages of the rotifer Philodina acuticornis, have all contributed to a knowledge of the requirements of different species under laboratory conditions, though extrapolation of their results to natural habitats is not always possible. The work of Lansing 1942a showed that there existed a difference in species response to the effects of total salt concentration in the rotifers Proales sp. and Rotifer vulgaris cultured on standardised Knop's medium. His study of the effects of pH value on the rotifer Euchlanis triquetra, which found that as pH value increased, so also did the length of the post reproductive period, are in agreement with a similar study conducted by Pray on the rotifer Philodina megalotrocha in 1959. That the species response to pH was similar in these two very different species is complicated by the criticism that both studies employed different buffers to obtain the different pH values throughout experiments. The response of different rotifer species in relation to their specific requirements in artificial and natural culture regimes can only be elucidated when standardised and aseptic culture procedures have eliminated the sort of variables that are too frequently found in the experimental culture conditions of many rotifer ageing studies. In

this present study an attempt was made to eliminate variability in culture procedures and to gain a knowledge of some at least of the species requirements in artificial culture conditions. This was done as a prerequisite to the experimental ageing studies that constituted the subject of this work.

INTRODUCTION TO MATERIALS AND METHODS

1. CHOICE OF CULTURE CONDITIONS

The following facets of rotifer culture regime were carefully considered during the early laboratory experiments: composition of culture medium, total salt concentration, pH value and buffers, choice of algal food regime, the temperature of cultures, and the storage of cultures without evaporative loss. Finally the methods of handling experimental rotifers and the methods of establishing sterile axenic rotifer cultures over the duration of experiments were also considered.

The choice of composition for culture media was based on the previous successful use on my part of a modified Knop's medium. This culture medium had been adopted by Lansing in his Ageing studies and consisted of 1 gm $Ca(NO_3)_2$, 0.375 gms $MgSO_4 \cdot 7H_2O$ and 0.375 gms KNO_3, diluted to a total salt concentration of 0.04%. It had the advantage of being simple in content (hence aiding the analysis of results where the relative concentrations of component ions were varied) and being readily controlled and standardised throughout use, unlike the variable grass and hay infusions that are often used for rotifer culture. The inorganic composition of the Knop's medium was also of great help in the maintenance of its sterility. Although the ratios of salts in Knop's medium were varied in some of the experiments described later, the ratios mentioned above were always adopted as a Control and are therefore always referred to as Knop's Control medium.

The total salt concentration of Knop's Control medium was chosen to lie well within the limits of salt tolerance for the particular species chosen for study. In the case of Mytilina brevispina var redunca, the tolerance limits lay between the values of 0.02% and 0.06%. At both of these values reductions in reproductive activity were evident, while above 0.06% total salt concentration a reduction in life-span occurred. The value of 0.04% total salt concentration was chosen because it lay well within the limits of salt tolerance for the species and because it had been previously used successfully by Lansing and King for closely related Monogonont species.

The pH value chosen for culture media was matched to the pH value of pond water at the time and level at which rotifers of the species Mytilina brevispina var redunca were collected. It was difficult to test the effects of pH on the rotifer species employed in this study as it proved impossible to produce a sufficiently wide range of pH change with the Sörensen's phosphate buffer adopted for experimental culture regimes. However within the range pH 6 to 8, produced by adding the same quantity of Sörensen's buffer, little effect was observed on longevity or egg production in this rotifer. The pH value 7.3 was maintained in culture media by the use of Sörensen's phosphate buffer, which satisfied the experimental requirement for a buffer that was non toxic and could be employed at a low concentration in large volumes of Knop's medium without loss of pH stability.

The algal species chosen as food regime was Chlamydomonas rheinhardtii. An algal rather than a bacterial food regime was preferred, as previous experience with bacterial regimes had shown that the toxic bi-products built up by bacteria in individual cultures of Monogonont rotifers had produced deterimental effects on the rotifers themselves, as well as pH instabilities in even well buffered culture medium (King 1967). Little success has so far been obtained in the field of rotifer culture, using monoxenic (synxenic) bacterial

regimes, although Meadow and Barrows (1969) achieved considerable success in the culture of the Bdelloid rotifer Philidina acuticornis when they used a dixenic bacterial regime consisting of Aerogenes aerobacter and Pseudomonas N6 based on dried grass infusion.

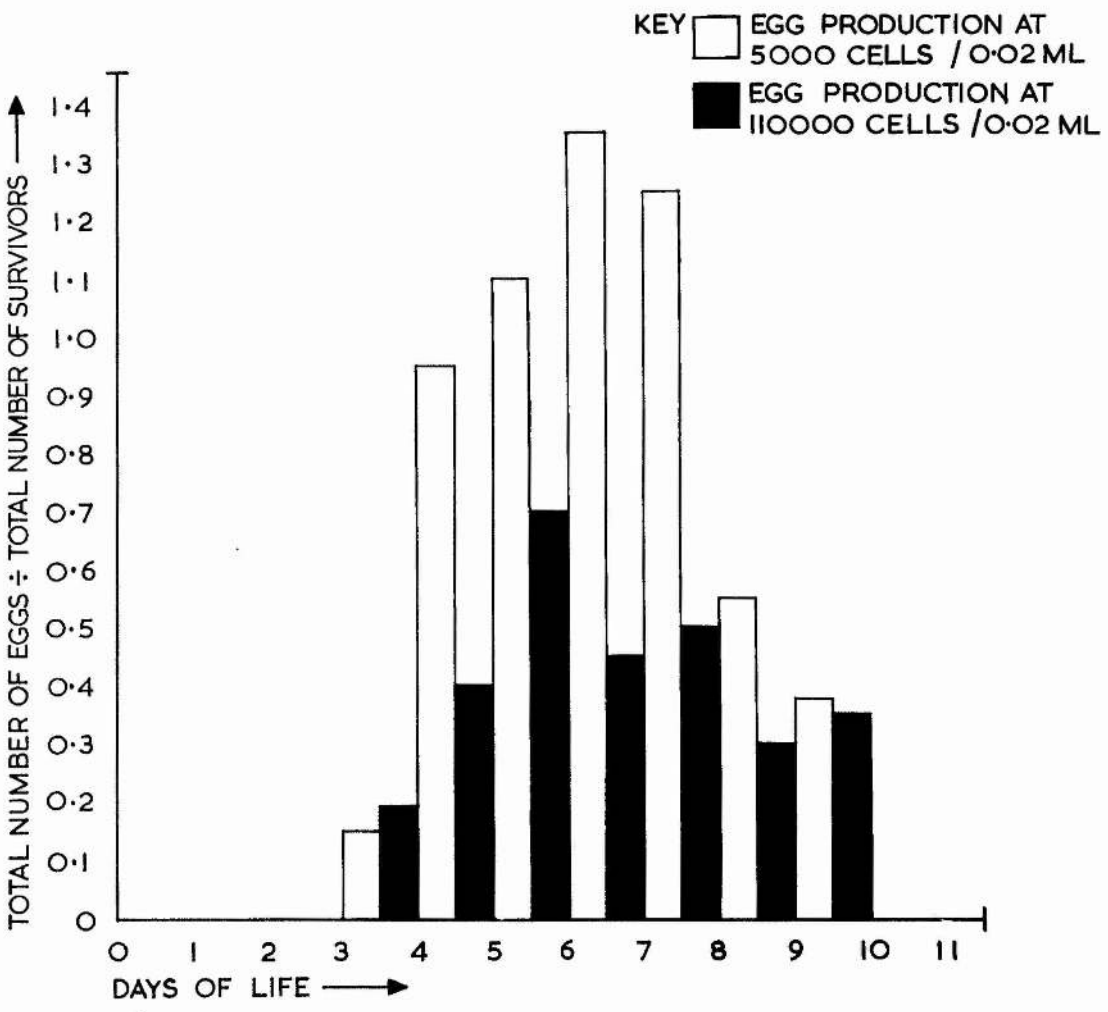
It was observed early in this study that the construction of the corona of Mytilina brevispina var redunca precluded the ingestion of cell aggregates. Unlike the situation commonly found in the Bdelloidea in the case of this Monogonont species the coronal musculature did not permit extension of the cingulum or of the coronal lip, a pre-requisite for forageing in cell aggregates to obtain cells for ingestion. This consideration influenced me in the choice of a motile algal food regime such as Chlamydomonas or Euglena sp., rather than a non motile form such as Chlorella sp. which will readily form aggregates and films in cultures. However trial experiments carried out with the motile alga Euglena viridis and Euglena gracilis revealed that the construction of the corona of Mytilina brevispina var redunca did not permit the ready ingestion of the elipsoid cells of these algae. Bearing in mind that Pourriot has shown that the most successful food regimes for the group Brachionidae appeared to be small spherically shaped algae rather than elipsoid forms, a species of Chlamydomonas namely Chlamydomonas iyengarii, which possessed extremely small spherical size was next tried as food regime. Although results proved very successful the culture of this alga in the long term proved to be complicated by resting phases during which large numbers of non motile cells were evident. An alternative algal species Chlamydomonas rheinhardtii which also possessed small spherical size but which did not enter into non motile phases in its life-cycle was adopted as food after a successful series of trial experiments. It should be emphasised that success of a food regime was estimated in terms of fecundity, rate of maturation, growth, general appearance and motility in rotifers.

Much attention was directed towards the long term culture methods employed for the algal species chosen as food regime. Initially Chlamydomonas rheinhardtii was obtained in a sterile liquid culture (axenic wild type strain 11/32C) from the Cambridge Botany School collection. Approximately 3 mls of the culture were inoculated into 50 mls of Bold's Basal medium enriched with 0.1% peptone and contained within a sterile plastic vessel. Cultures were maintained under flourescent light at ambient temperature before being slowly adapted to temperatures above ambient in parallel with the rotifers in the sterilizing group (see later). All cultures were aerated in a sterile air cabinet for 3 minutes daily, and this procedure was accompanied by gentle agitation of the culture medium in an attempt to preserve algae in a fully motile condition at all times. Every 10 days approximately 3 mls of a suspension containing algae that had been spun down at 1200 rpm for 3 minutes and resuspended and spun down again in a fresh volume of Bold's supplemented culture medium, were inoculated into a fresh 50 ml volume of sterile supplemented medium in a sterile plastic vessel.

Before algae were employed in experimental cultures the following culture procedures were adopted. Approximately 8 mls of the algal medium were spun down as before and resuspended in a large volume of Beijerinck's medium supplemented with 0.1% peptone, before being spun down again and resuspended in 50 mls of Beijerinck's supplemented medium in a sterile flask. After a further 10 days this procedure was repeated, only this time algae were transferred to 50 mls of unsupplemented Beijerinck's medium where after a further week they were ready for experimental use, after being spun down, washed twice in the appropriate Knop's Control medium and transferred in Knop's Control medium to the appropriate culture, after the concentration had been monitored by micronephelometer (Type 236 Scientific Furnishings Poynton, Cheshire) and Thoma haemocytometer. If necessary the concentration was adjusted to the correct experimental level by dilution with the appropriate Knop's medium. The technique of estimating algal concentration by

micronephelometer required that only fully motile suspensions were used, since sedimenting non motile fractions would give rise to inhomogeneities in the suspension and hence errors in the estimation of light scattering. This, together with the species requirement for a fully motile food medium, was the reason for the algal culture method described. The transfer of algae from supplemented Bold's culture medium to supplemented then unsupplemented Beijerinck's medium, was aimed at extending the log or fully motile phase of culture development, together with the gradual lowering of complexity and total salt concentration of the algal culture medium itself, as an adaptation procedure before algae were transferred to the simple dilute Knop's medium adopted for experimental cultures. It had been found that while the complex Bold's Basal medium provided excellent conditions for the cultivation of long term algal stocks, a very rapid log phase of development took place at the experimental temperature finally employed ($24^{\circ}C$), even if the volume and concentration of inoculate was low. This rapid and short log phase of development was always followed by a slight fall-off of the numbers of motile cells at the stat phase of culture development, hence fully motile algal suspensions could only be obtained from cultures changed with inconvenient frequency every 3 days. By contrast, unsupplemented Beijerinck's medium provided a comparatively long log phase of development during which motile algal samples could be obtained for experiments. Furthermore, the simplicity of this medium seemed to pre-adapt algae to the dilute Knop's media before transfer, thereby causing no temporary reduction in motility when the monitored algal supplement was added in the appropriate Knop's medium to experimental cultures. It should be emphasised that although the identical culture method was always carried out on algal cultures destined for use in experiments, all long term culture of algal stocks was carried out on the complex Bold's Basal medium, when the non motile sedimenting fraction could be eliminated at each culture change.

The selection of the concentration of food was based on trial experiments in both individual and mass cultures. It was found that at food levels in excess of 100,000 cells per 0.02mls of Knop's Control medium, egg-laying in both individual and mass rotifer cultures was reduced, with the maxima of egg-laying of any population below index 0.75 (egg index being the total number of eggs


KEY
EGG PRODUCTION AT 5000 CELLS / 0·02 ML
EGG PRODUCTION AT 110000 CELLS /0·02 ML
TOTAL NUMBER OF EGGS ÷ TOTAL NUMBER OF SURVIVORS
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS OF LIFE

Histogram 1

laid divided by the total population that laid them) See Histogram 1. The same effect was observed in individual and mass rotifer cultures containing less than 500 cells per 0.02 mls of Knop's Control medium, and at concentration levels below 100 cells per 0.02 ml Knop's Control medium, individual rotifer cultures displayed very little egg-laying at all. Single and mass cultures that were supplemented between the values of 4000 and 8000 cells per 0.02 ml Knop's Control medium displayed stable and similar longevity values over 5 generations of experimental culture (30 individuals being assessed at each generation), with similar and stable egg-laying maxima of at least one egg per individual per day. A food value was chosen between these two values that provided an excess of food required by an individual rotifer, but was of itself low enough in concentration to be unlikely to greatly alter the composition of the 0.02 ml culture droplet during the time the culture was established (See experimental results on ^{45}Ca food culture). For this reason a value of 5000 cells per 0.02 mls of culture medium that lay towards the lower limit of this range was chosen for all experimental cultures throughout this study.

Aseptic conditions prevailed throughout the culture of algae and rotifers. Rotifers were initially sterilized by a modified Hetherington technique described later, and all media were sterilized monthly by filtration method. Glassware was sterilized by dry air sterilization at 170°C for 3 hours after it had been acid cleaned and then rinsed for a week in distilled water. All disposable plastic materials employed during the course of experiments had been previously sterlized by gamma ray irradiation.

The selection of culture temperature was based primarily on a consideration of the upper lethal temperature of the species and on the convenience of an approximately 8 or 9 day life-span for the purposes of experimental procedures. While temperatures of up to 35°C could be tolerated with correct adaptation, there were signs of reduced egg-laying and distress at temperatures above this value. The selected temperature of 24°C produced a life expectancy of between 8 and 9 days (the required life-span), and was itself well below the upper critical range for the species.

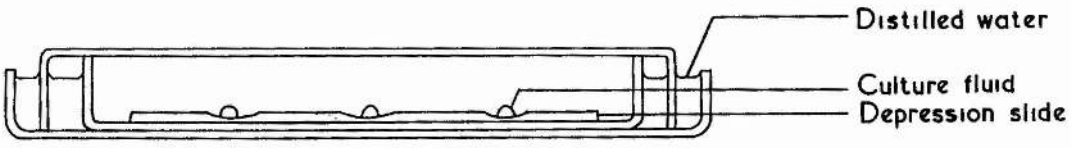


Diagram 2

Procedures for the storage of experimental cultures were designed on the basis of trial experiments involving a variety of water-sealed containers. The most satisfactory storage container consisted of an inner tray bearing the depression slides, that was itself contained within a 6 inch diameter inverted crystallizing dish. The annulus of the inverted dish which included an annulus surrounding an inner tray and an annulus surrounding the lid, provided a double water seal that effectively prevented any detectable evaporative loss occurring in the ten 0.02 ml culture droplets on each tray within a period of 24 hours. It should be mentioned that the surface area of each droplet was kept to a minimum by coating each depression slide with silicone (slides were rinsed for several days after the coating process) which caused droplets to round up and not spread unevenly over the slide. A diagram of the storage method is shown (Diagram 2). Mass cultures of rotifer stocks were stored in 4 cm petri dishes on which the fluid level was marked with a hairline in order that evaporative losses could be made good by the addition of sterile deionised water.

All rotifers in this study were handled by means of fine glass capillaries made from Pasteur pipettes. The capillary tip which was drawn out in a flame and smoothed off with ground glass chips was always constructed to be of slightly smaller size than the rotifers handled. In this way rotifers were never actually sucked into pipettes during transfer, but were affixed to the capillary tip by suction and manipulated between cultures in this manner. Although it required practice, this procedure eliminated the transfer of significant volumes of fluid with the rotifers themselves, and also eliminated the risk of rotifers adhering to the inside of the glass capillary.

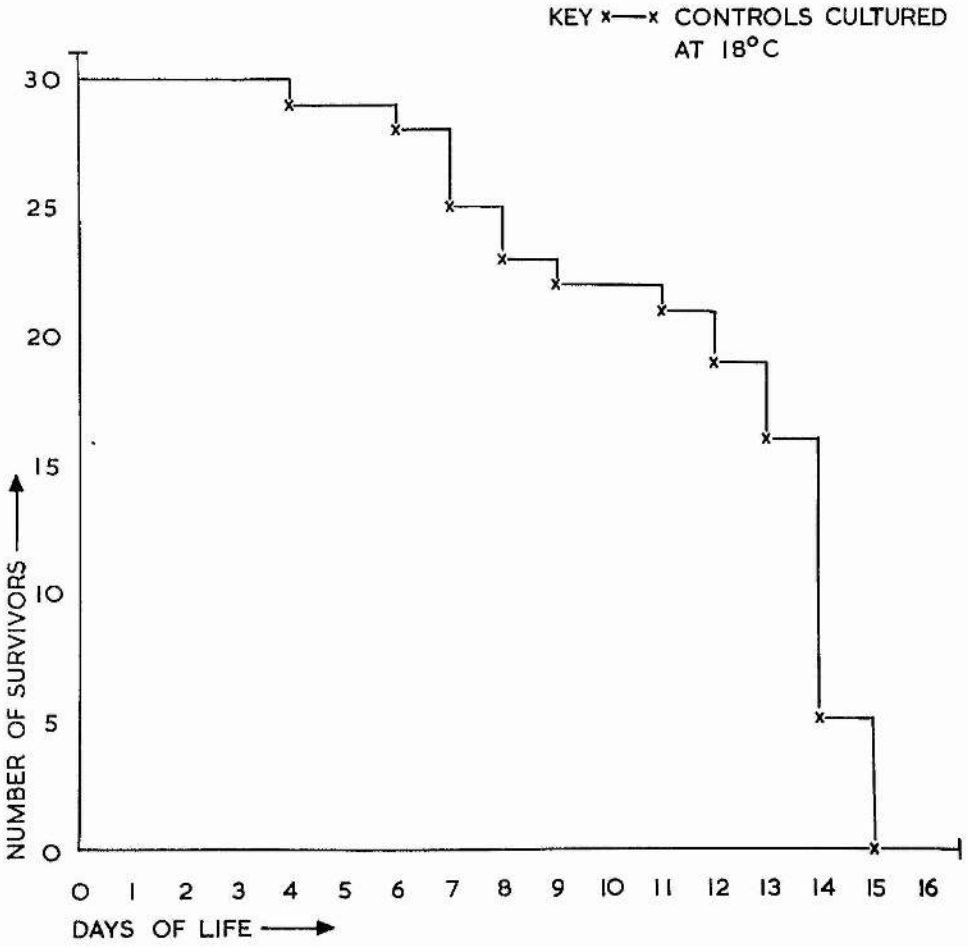
INTRODUCTION TO MATERIALS AND METHODS

2. ADAPTATION PROCEDURES TO STERILE ARTIFICIAL CULTURE

4 rotifers of the species Mytilina brevispina var redunca were taken from a local pond and cultured in the laboratory in 4 cm petri

dishes containing 5 mls of pond water at 6°C. This was the temperature of the pond water at the time and depth at which rotifers were removed. The temperature of the pond water cultures was allowed to rise by 1°C in 24 hours up to room temperature, while rotifers and their progeny were transferred by fine capillary pipette to fresh 5 ml aliquots of pond water on alternate days. After 2 days spent at room temperature (about 16°C), 20 freshly hatched rotifers (hatchlings) and the same number of eggs were washed individually in separate 5 ml volumes of sterile Knop's Control medium buffered to pH 7.3, and a population of ten, consisting of five eggs and five hatchlings transferred to each of four 4 cm petri dishes containing 5 mls of sterile Control medium supplemented with a measured quantity of motile algae of the species Chlamydomonas rheinhardtii (Cambridge Botany School number 11/32C). The algae which were sterile on arrival were cultured at room temperature on sterile unsupplemented Beijerinck's medium, and spun down at 1200 prm for 3 minutes and twice resuspended in fresh large volumes of Control medium before being dispensed in equal concentrations and volumes of Control medium, to each dish. The algal concentration, was 5000 $\pm$ 50 cells per 0.02 ml culture medium. All four dishes were maintained under flourescent light and their contents aerated and gently agitated for 3 minutes daily in a sterile air cabinet to help prevent the formation of surface filmsof palmelloid cells. The fluid level in each dish was marked with a hairline in order that any evaporative losses that took place from the closed dishes could be made good by the addition of sterile deionised water, and the total salt concentration in each dish thus preserved.

After a further 6 days, one young rotifer was selected randomly from each of the four petri dish cultures and transferred individually to separate 150 cc glass tanks containing 15 mls of sterile Knop's Control medium. A modification of Hetheringtons 1934 sterilization procedure for protozoans was then carried out by noting the place at which the animal had been introduced into the tank, and collecting it after it had migrated to a place far distant. In all cases this occurred within 5 minutes. One of the four washed rotifers was then placed in each of four 4 cm petri dishes containing 5 mls of sterile Knop's Control medium, supplemented with the same quantity of sterile motile algal suspension as before. On alternate days the


KEY x—x CONTROLS CULTURED AT 18°C
NUMBER OF SURVIVORS
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS OF LIFE

Graph 1

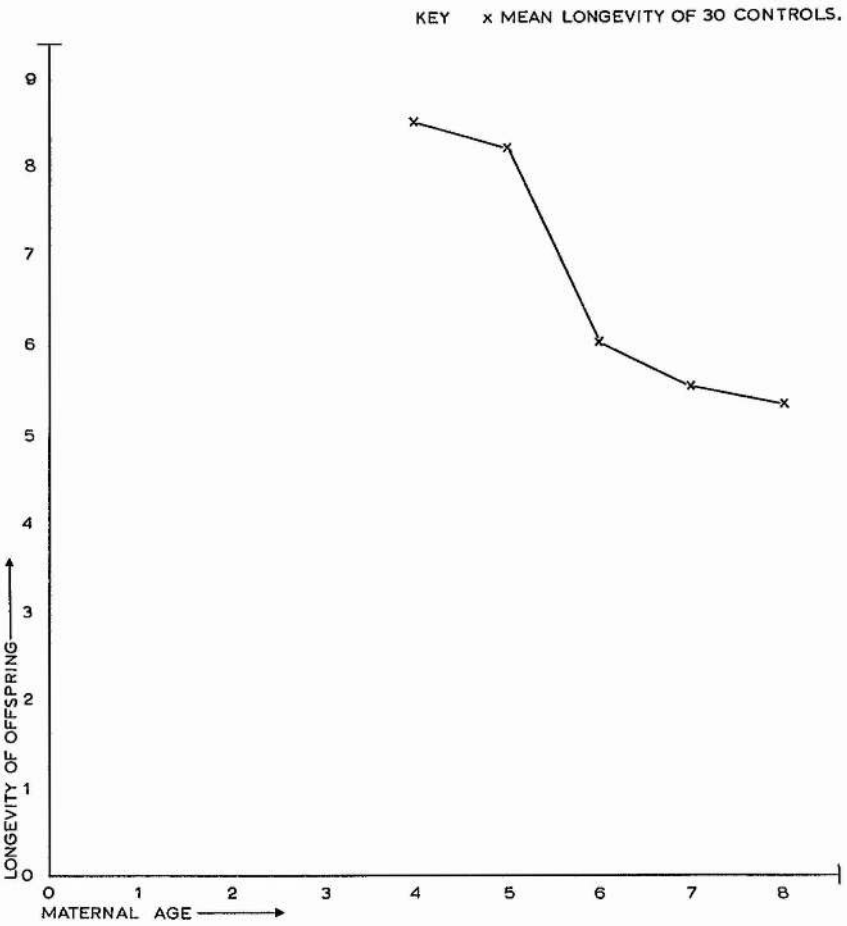
rotifers were washed by the modified Hetherington procedure and the culture temperature raised by 1°C until it reached 18°C. After 5 days one healthy young rotifer was selected from each culture, washed and transferred to one of four fresh 5 ml cultures, and maintained at 18°C in a water cooled incubator for a further week when the cycle was repeated. All unhatched eggs were washed in 15 mls of sterile Control medium and the use of toxic disinfectants such as kanamycin sulphate and sodium cephalothin (Meadow and Barrows 1969) was avoided.

All the discarded progeny from the dishes were transferred to a single mass culture consisting of 5 mls Knop's Control medium, and 30 young rotifers that hatched in a single day were removed to a watch-glass containing 0.20 mls of Knop's Control culture medium supplemented with the same food concentration as used before (i.e. 5000 cells per 0.02 ml of culture medium). The watch glasses were stored in water sealed containers at 18°C in an incubator and rotifers were transferred to fresh watch glasses daily, when the number of eggs laid and the number of individuals surviving were recorded, in order that a survival curve could be plotted for the original 30 individuals, Graph 1 (all eggs and young were discarded at each culture change). A mean life-span of approximately 12 days was obtained for this experimental population, and it was decided on the basis of this result to raise the temperature of the sterilizing group of cultures to 24°C over the period of 3 weeks, when a survival curve was again plotted in the same manner for a mass culture of 30 Control cultured rotifers that hatched on a single day (Graph 1). The mean longevity of this group was approximately $8\frac{1}{2}$ days which was considered a convenient length of life for the procedures of this present study.

After 10 days, the sterility of the animals subjected to the modified Hetherington washing procedure was tested by inoculating progeny discarded at the 5th day into 5 ml samples of a sterile 1% peptone solution. Small amounts of washing medium and vacated culture medium were also tested in this manner. After approximately 3 months, discarded progeny of one of the four sterilizing groups of cultures failed to produce any bacterial growth in the peptone.

Likewise, washing medium and vacated culture fluid inoculate also proved negative. The sterile healthy young rotifer selected from this culture was to be the original parent of all experimental rotifers adopted in this study. It was transferred to a 0.02 ml droplet of Knop's Control medium supplemented with 5000 cells of algal food regime on a depression slide that was maintained in a closed petri dish saturated with water vapour. The petri dish was stored at 24°C under flourescent light in an incubator. The culture droplet was changed daily, when all progeny were maintained in similar individual cultures on depression slides and finally transferred to a Constant Temperature Room running at 24°C ± 0.2°C for the orthoclone selection procedures leading to the first experiments. Frequent tests were carried out throughout the course of experiments to ensure that rotifers and all culture media remained sterile.

The purpose of the foregoing procedures was to adapt rotifers of the species Mytilina brevispina var redunca taken from a natural habitat to the artificial culture conditions of the laboratory, over a reasonable time period. At the same time as the adaptation procedures were taking place, washing procedures were carried out in order that a single sterile individual could be selected to form the parent of all experimental progeny. Throughout the time adaptation procedures were occurring, observations were frequently made in rotifer cultures for any signs of mixis and the accompanying formation of dormant eggs. It is important to stress that no signs of this process were evident during initial laboratory culture or at any other time during the course of this present study, which is in agreement with the 1909 survey by Brauer where no record of mixis is to be found for the var redunca of the species Mytilina brevispina.


KEY x MEAN LONGEVITY OF 30 CONTROLS.
LONGEVITY OF OFFSPRING
0
1
2
3
4
5
6
7
8
9
MATERNAL AGE

Graph 2

TABLE 2.

MATROCLINY RESULTS

30 rotifers employed for each Group.

DAY GROUP	MEAN LONGEVITY	TOTAL EGGS
4 day	8.5 days S.E. ± 0.3 days	150 eggs
5 day	8.2 days S.E. ± 0.3 days	102 eggs
6 day	6.0 days S.E. ± 0.3 days	68 eggs
7 day	5.5 days S.E. ± 0.3 days	48 eggs
8 day	5.3 days S.E. ± 0.3 days	28 eggs

INTRODUCTION TO MATERIALS AND METHODS

3. MATROCLINOUS VARIATION IN THIS SPECIES AND THE METHOD USED TO ELIMINATE THIS VARIATION IN EXPERIMENTAL CULTURES.

Before selection procedures were carried out a series of experiments were undertaken to test the influence of matroclinous deviation in this species with respect to such characteristics as mean longevity and egg production. The offspring of mothers originally descended from a single parent, whose egg-laying ages ranged between 4 and 8 days inclusively, were cultured individually in 0.02 mls of Knop's Control medium at 24°C (see Ion ratio Experiment Materials and Methods Page 36 for full culture conditions) such that a group of 30 individuals were derived from mothers at each age. The descendants were all 1st generation individuals, and were isolated into separate cultures on being laid. Thereafter, only groups of 30 rotifers that hatched from eggs within a period of 6 hours, 1½ days after deposition, were used to represent mothers at each of the experimental ages. If by chance a mother laid two eggs on the same day both eggs were discarded from the experiment to avoid confusion. No delay in hatching was recorded in any of the eggs of the mothers of different ages, but experimental results did reveal fundamental differences in mean survival time and total egg production between groups, see Table and Graph 2. Although little difference in mean longevity was evident between the 4 and 5 day rotifers, the rotifers derived from 6 day old mothers showed a reduction in mean longevity of approximately 2½ days, 29% of the maximum longevity shown by any of the experimental groups. The 7th and 8th day offspring showed further mean longevity reductions, to bring the total reduction in mean life-span of the oldest rotifers derived from 8 day old mothers to 3.2 days (37%). On the basis of Lansing's definition of orthoclones in 1948, and it must be emphasised that the rotifers employed in this present study represented only one generation of selection and were not clones, the 6th, 7th and 8th day offspring in this experiment could have produced geriaclones while further generations of selection would have been required to establish whether or not the 4th and 5th day offspring could have given rise to isoclones or pediaclones. It is interesting to note that onset

of egg-laying occurred on the same day in each of the experimental groups, and though the total number of eggs diminished with the accompanying decreases in mean longevity, similar egg production maxima were evident during the reproductive period. The observations were restricted in this experiment to the first generation of descendants, and doubtless more deviation between groups would have become evident at later generations. The overall impression gained from this experiment was that this species of rotifer exhibited strong matroclinous deviation, since considerable variation in mean longevity occurred at only the first generation of selection of offspring.

Matrocliny was first recorded by Jennings and Lynch for the rotifer Proales sordida in 1928. These authors sought to explain the large amount of variation in populations of this rotifer with regard to longevity, egg production, rate of development and egg size. In the absence of genetic variation between individuals (Proales sordida reproduced in their experiments only by parthenogenesis) Jennings and Lynch set out to establish that in a non variable environment maternal age at egg-laying, was the source of the intrinsic differences in their experimental populations. To do this observations were carried out on individuals that were the second generation descendants of mothers of different ages, and although environmental variables were not satisfactorily controlled during experiments by modern standards, their results supported the broad conclusion that when an individual aged its progeny became more variable with respect to fecundity, longevity and rate of development. Lansing on the basis of the findings of Jennings and Lynch, reasoned that orthoclones established from parents of different ages over the course of several generations should amplify the effects of matroclinous variation. In 1947, 1948 and 1954, he investigated such variation in orthoclones of the rotifer Philodina citrina, duly establishing in each paper that the mean longevity of young 5 day orthoclones of this species increased, while that of old 16 day orthoclones decreased, over several generations of selection. The use of the term "pediaclone" for the former orthoclone and "geriaclone" for the latter orthoclone has already been mentioned in the Introduction, as has also Lansing's argument that a

TABLE 3

(Data from Series 2 and 3 Lansing 1947)

	5 D A Y			8 D A Y	
GENERATION	MLS	RATIO	GENERATION	MLS	RATIO
0	24.2	-	0	20.4	-
1	22.8	0.94	1	23.1	1.13
2	23.5	1.03	2	22.5	0.97
3	19.2	0.82	3	22.6	1.00
4	24.3	1.27	4	21.5	0.95
5	24.5	1.01	5	20.8	0.97
6	24.3	0.99	6	19.6	0.94
7	27.8	1.14	7	14.9	0.76
8			8	0	0
MEANS	23.8	1.03		18.4	0.84

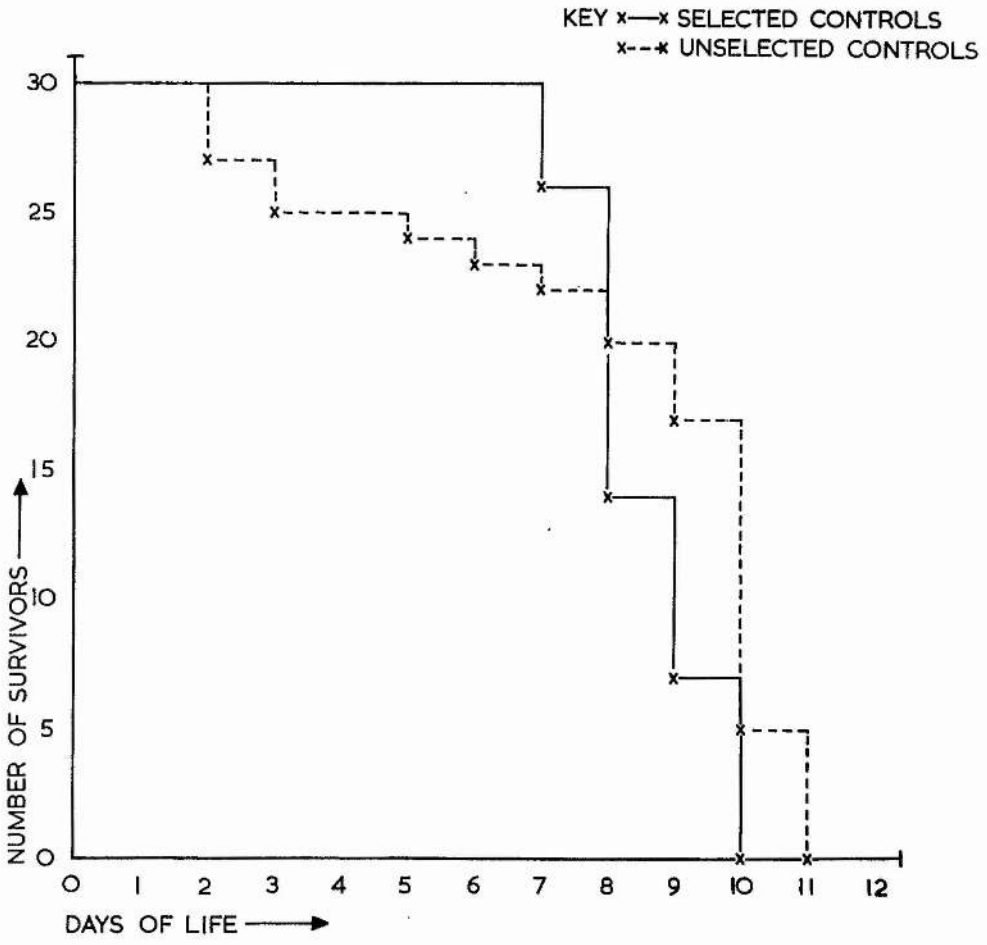
transmissable and cumulative cytoplasmic factor that appeared at the time of cessation of maternal growth was responsible for the decreasing longevity in geriaclones. Furthermore, this factor was related in 1947 to the increase in calcium content with age that was dealt with in his 1942 paper. Philodina citrina was a well chosen species for demonstrating the absence of the postulated ageing factor in orthoclones derived from growing mothers, since this species unlike the species chosen for this present study begins its reproductive activity during the final stages of active growth in size. On the basis of only one generation of selection Mytilina brevispina var redunca showed no significant reduction in longevity in the case of rotifers established from mothers 1 and 2 days after they had completed their growth in size. It would have required further generations of selection in this rotifer to ascertain the exact point in life at which the change occurred from pediaclone to geriaclone, however it seemed likely that this change would be before the 6th day, since after this day offspring would have shown a substantial reduction in number at each generation of selection, with 7th and 8th day individuals becoming extinct after the first generation of selection in the experiment. It is interesting to note that Lansing discovered in his orthoclone studies in 1948, that onset of egg-laying tended to occur at earlier ages in geriaclones and at later ages in pediaclones, a result which was not evident in the case of Mytilina brevispina var redunca, where on the basis of only one generation of selection, onset of egg-laying occurred simultaneously in all individuals.

It is possible to compare Lansing's experimental results with the predictions of his hypothesis of a transmissable cumulative ageing factor. Table 3 shows the results of a 5 day pediaclone and 8 day geriaclone of the rotifer Philodina citrina (data from series 2 and 3 Lansing 1947). The ratio of the mean life-spans between succeeding generations are entered in the Table alongside the mean life-span values themselves. Any cumulative ageing factor that is transmitted through succeeding generations of orthoclone selection should produce a steady pattern of increase or decrease above or below the value of unity, between generations of orthoclones. No such consistency of pattern is evident between generations, although the mean values for each orthoclone are of the expected magnitude and direction, with levels above unity being obtained for the pediaclone and levels below being obtained for the geriaclone. Indeed up till the 6th generation

in each orthoclone there is no evidence of changing life expectation. This apparently contradictory evidence for Lansing's postulated link between the observed effects of matrocliny and the cumulative transmissable action of an ageing factor related to increased levels of calcium in tissues, may have been the result of errors incurred in the small sample size of experimental populations.

Recently King has reported matroclinous variations in longevity and egg-laying for the rotifer Euchlanis dilatata. He found that the mean survival time of a young (1.5 day) orthoclone was 4.4 days, as compared with the 3.1 day survival time obtained for an old (3 day) orthoclone, both orthoclones being selected over 10 generations. This difference was significant at the 1% level of variance as was the difference between egg totals for each orthoclone, on average 4.8 eggs for a young orthoclone female per lifetime, and 2.7 eggs for an old orthoclone female per lifetime. Furthermore, a different and less variable egg size pattern for mothers of different ages than was found by Jennings and Lynch in 1928 in the rotifer Proales sordida, has been recently dicovered by King for the rotifer Euchlanis triquetra reared on a standardised Knop's medium. This result is of particular interest, as it demonstrates that matroclinous deviation could differ interspecifically within the entire phylum.

The matroclinous deviation previously discussed in the rotifer Mytilina brevispina var redunca, necessitated a selection procedure in the case of experimental populations to eliminate this source of variability between individuals. In this study, a 4 day orthoclone was always adopted in experimental regimes, and this orthoclone was always selected from mothers that were themselves derived from a single ancestor. Because of the possibility that such an orthoclone could show variations between generations, in the manner for instance of Lansing's orthoclones, it was important in the selection procedure to maintain the 4 day orthoclone for the same fixed number of generations in all cases before use in experiments. Three generations of selection were carried out in this study, in case a single generation of selection was not sufficient to completely homologise all experimental individuals with respect to the maternal age factor.


KEY x—x SELECTED CONTROLS
x--x UNSELECTED CONTROLS
NUMBER OF SURVIVORS
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12
DAYS OF LIFE

Graph 3

The selection procedure was always carried out with rotifers that were individually cultured in Knop's Control medium. However, in the Ion Ratio Experiment (Materials and Methods Page 38), where two additional media were used that differed in ionic balance to the Knop's Control medium, individuals at the first generation of orthoclone selection that were destined to be cultured in either of these two differing media, were transferred to the appropriate medium of the two in which they were to be cultured during the experiment. This ensured that mothers actually laid their eggs in the correct experimental medium, and gave some prior adaptation to the individuals that were cultured in the two non Control media of the experiment. The effectiveness of the selection procedures carried out to homologise experimental populations with respect to the matroclinous factor may be judged from the two survival curves shown, one for a selected group of 30 rotifers cultured individually on Knop's Control medium at 24°C (Graph 3), and the other from a randomly chosen unselected group of the same number cultured under the same conditions. It can be seen that the standard mean longevity error for the selected group ($\pm$ 0.17) was significantly lower than that for the unselected population, ($\pm$ 0.48) a 65% reduction in error over 3 generations. This difference was largely due to increased mortality in early life in the unselected group, presumably due to the fact that these individuals were derived from old mothers of old clones. It is interesting to note that the unselected Control survival curve for mass cultured individuals at 18°C, also shows a similar pattern of increased early mortality.

In many previous rotifer studies there has been little evidence of attempts to standardise culture conditions. Non standard culture media such as grass and hay infusions have often been employed, and in many cases these were used unsterilized. Temperature has been allowed to vary over more than 1°C total variation, and pH has often only been maintained at the expense of using large and variable amounts of different buffers. In this work a standard modified Knop's medium was employed for all experiments, and bacterial variables have been eliminated by culturing rotifers under aseptic

conditions. All experiments have been conducted in a Constant Temperature room that maintained total temperature variation in cultures within a range of less than 0.5°C. The pH of cultures has been maintained by means of small quantities of a standard and non toxic phosphate buffer (Sorensen's). Because mass culture conditions are often difficult to control and standardise owing to rapid population increases and the introduction of such variables as stress and competition, all rotifers in this work have been cultured individually in 0.02 ml droplets containing a fixed food regime (monitored by micronephelometer and haemocytometer). Finally, because matroclinous variation was evident in the species of rotifer under investigation, all rotifers that had been initially derived from the same single parent were homologised with respect to maternal age by the selection of a 4 day orthoclone over three generations for all experiments.

MATERIALS AND METHODS

ION RATIO EXPERIMENT

Rotifers were individually cultured under aseptic conditions in the wells of depression slides contained within inverted water-sealed crystallizing dishes. In addition to a 0.02 ml volume of modified Knop's medium supplemented with a standard quantity of algal suspension of the species Chlamydomonas rheinhardtii (5000 cells $\pm$ 50 cells) monitored by micronephelometer and haemocytometer, each well contained a single 4 day orthoclone rotifer of the species Mytilina brevispina var redunca. The algal component of each culture had been cultured on the simple Beijerinck's medium before use, although algal stocks were maintained on the more complex Bold's Basal medium supplemented with 0.1% peptone. All depression slides were stored under flourescent light (mean incident light 300 ft candles), in a constant temperature room at 24°C ($\pm$0.2°C), and all cultures were changed once every 24 hours during which period no volume changes in excess of 0.0002 ml could be detected in the culture droplets.

TABLE 4

	HIGH CALCIUM	CONTROL CALCIUM	LOW CALCIUM
SALT CONTENT	1.5 gm. $Ca(NO_3)_2$ 0.125 gm. KNO_3 0.125 gm. $MgSO_4$	1.0 gm. $Ca(NO_3)_2$ 0.375 gm. KNO_3 0.375 gm. $MgSO_4$	0.5 gm. $Ca(NO_3)_2$ 0.75 gm. KNO_3 0.5 gm. $MgSO_4$
SALT CONCENTRATION	0.04%	0.04%	0.04%
Ca CONCENTRATION PER 0.02 mls	7.2×10^{-6} gms	4.8×10^{-6} gms	2.4×10^{-6} gms
RATIOS Ca CONCENTRATION	3	2	1
pH	7.3	7.3	7.3

Three modified Knop's media each buffered with Sörensen's phosphate buffer to pH 7.3 and having a total salt concentration of 0.04%, were used as culture media for 3 populations of 30 individually cultured rotifers. The first medium was employed as a Control (see Table 4), while the individual salt concentrations of the other two media were adjusted to give a Low calcium - high potassium and magnesium balance and a High calcium - low potassium and magnesium balance respectively. The ratio of calcium salt concentration between High, Control and Low calcium media followed a 3:2:1 pattern, though the total concentration of salts was the same in each medium. Each of the 3 rotifer populations was scored daily for numbers of survivors and numbers of eggs produced, while each rotifer was measured twice daily during the growth period between widest points on transverse and longitudinal body axes, using an eyepiece graticule. It was not necessary to use methyl cellulose or anaesthetics during growth measurements in this species. From the data so obtained could be plotted a survival graph, Graph 4, a histogram, Histogram 2, of the average number of eggs laid per individual per day, and a growth curve, Graph 5, for each of the 3 experimental populations.

A 4th population of 30 rotifers was cultured on Knop's Control medium, but was subjected to 45 second immersions in 0.04 ml volumes of a 0.5% solution of sodium citrate on alternate days starting on day 3 of life. The citrate treated rotifers were washed twice in 0.04 ml volumes of Knop's Control medium before being transferred back to fresh Control cultures.

RESULTS

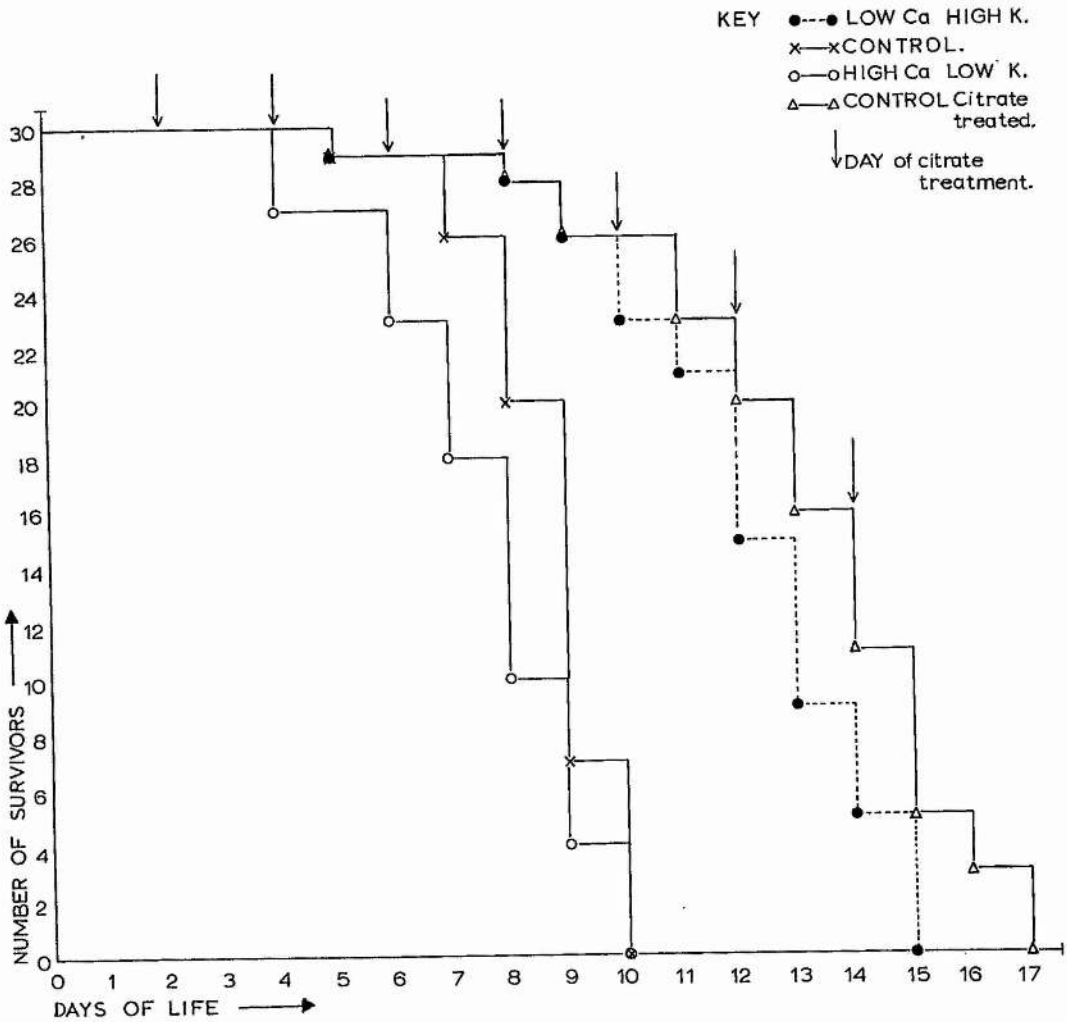
ION RATIO EXPERIMENT

No deaths were recorded in any of the four experimental populations during the period of growth and maturation, days 1 to 3 inclusive (see Graph 4). A major contribution to this absence of adolescent mortality in survival graphs was undoubtedly the selection over three generations of a 4 day orthoclone for all experimental populations.

TABLE 5

ION RATIO EXPERIMENT

POPULATION	MEAN LONGEVITY	INCREASE IN LIFE EXPECTANCY	TOTAL EGGS LAID
CONTROLS	8.7 days S.E. $\pm$ 0.2 days	-	136 eggs
HIGH Ca	7.6 days S.E. $\pm$ 0.2 days	- 12.6%	98 eggs
LOW Ca	12.2 days S.E. $\pm$ 0.3 days	40.2%	210 eggs
Na CITRATE	13.2 days S.E. $\pm$ 0.3 days	51.7%	223 eggs


KEY
LOW Ca HIGH K.
CONTROL.
HIGH Ca LOW K.
CONTROL Citrate treated.
DAY of citrate treatment.
NUMBER OF SURVIVORS
DAYS OF LIFE
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Graph 4

The first death in the Control group of rotifers was that of a single rotifer at day 5, the day following full growth and maturity. Thereafter a consistent decline in population number did not begin until day 7, when three rotifers (10% of the original population number) died. The remaining number of Control individuals all expired within the following three days, the last mortalities in this group being recorded on day 10. The highest mortality figure for any one day, was recorded on day 9, when 13 rotifers (43% of the original Control group) died. The overall form of the Control survival graph approximated more closely to a rectangle than did the graphs of the other three groups. This indicates that the Control culture conditions approached an optimum for this species, and that the Control population was uniformly susceptible to death.

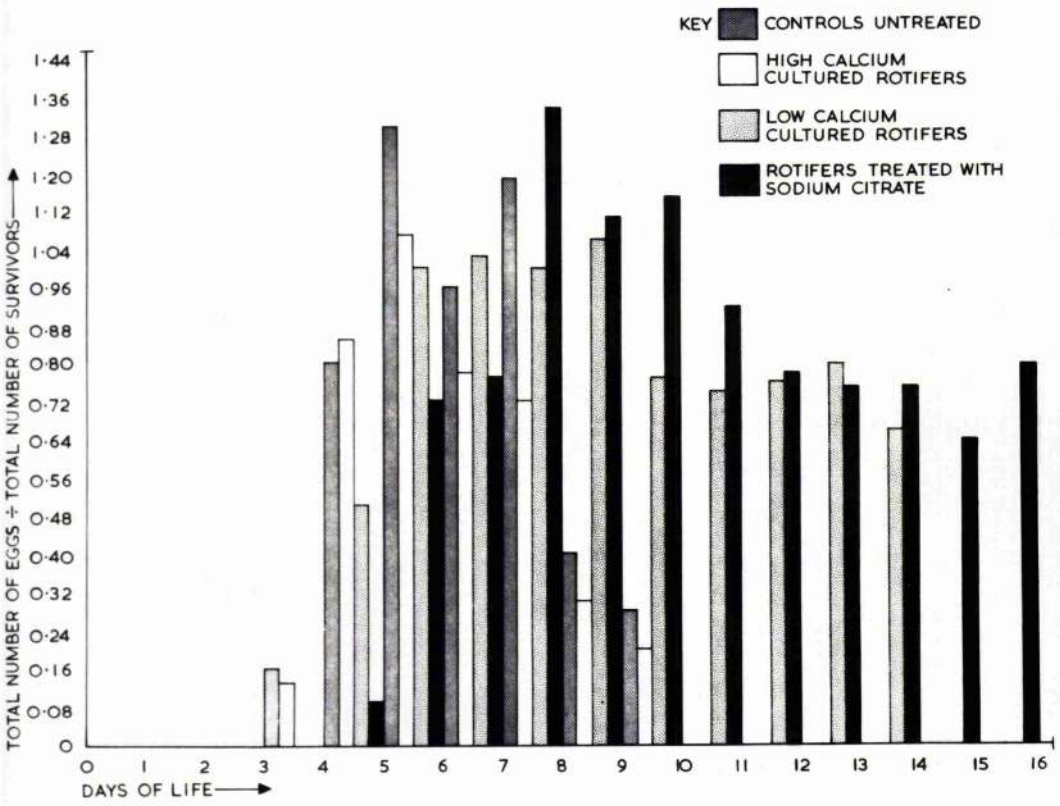
The survival graphs of the other three populations showed overall differences in their pattern of survival from the Control population. The onset of mortality in the High calcium group, for instance, at day 4 when three rotifers died, occurred a day earlier than the onset of mortality in the Control group or indeed in any of the other groups where only a single rotifer died. Thereafter, a consistent decline in the High calcium group did not begin until day 6, 23 rotifers being recorded alive on this day compared with 29 in each of the other three groups. This decline occurred at a faster rate than did the decline in numbers for the Control group, such that on day 8 when there were only ten High calcium rotifers surviving, twice that number of survivors were recorded in the Control group, and all but two of the original population were still alive in the Low calcium and Citrate treated groups. The final mortalities in the High calcium group were recorded on day 10 (the same day that the Control group expired), and the overall mean longevity value was 7.6 days (see Table 5), 1.1 days below the 8.7 day mean longevity value of the Control population.

On day 10, when both the Control and High calcium groups expired, 26 survivors (87% of the original population number) were recorded in each of the Low calcium and Citrate treated groups. Thereafter both groups declined gradually and at approximately the same rate until day 12, when the Low calcium group began to die out at a faster rate.

On day 15 when the last Low calcium rotifers died, five rotifers were still alive in the Citrate group corresponding to 17% of the original population number. These five survivors died within the next 2 days, three of them dying on the day the group expired, day 17. The mean longevities of the Low calcium and Citrate groups were 12.2 and 13.2 days, indicating an increase in life expectancy of 40.2% and 51.7% respectively in each group (see Table 5). The overall form of the survival graphs for the Low calcium and Citrate treated populations did not approximate as closely to a rectangular form as did the graphs of the other two groups. This may have indicated that the low uptake or removal of calcium reduced the effect of some calcium dependent cause of death, to which rotifers in these groups were equally susceptible, and animals then died from one or more causes of death resistance to which was not so homogeneously distributed throughout the two populations.

Egg production in each of the four experimental groups was expressed as an index calculated by dividing the total number of eggs laid on any one day by the total number of survivors. In this way, any slight change in the average rate of egg-laying per population could be immediately recognised on a histogram. It should be emphasised that to avoid confusion, any individual that expired on any one day, but had nevertheless laid an egg on that day which had been counted in the day's egg total, was added to the number of survivors recorded for that day in the calculation of the egg index.

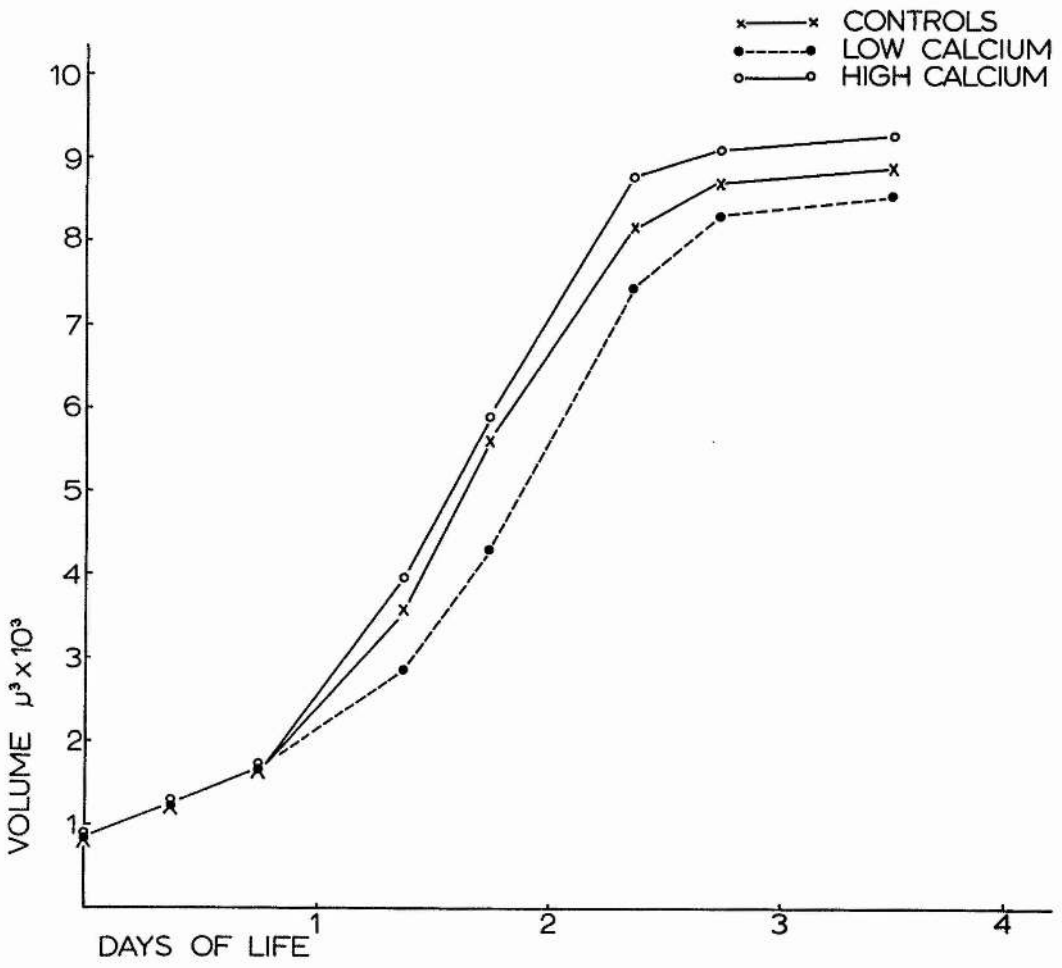
The Control population began egg-laying at day 3, when five rotifers in the group laid a single egg (see Histogram 2). Thereafter, the rate of egg production showed a rapid and consistent increase up till day 7, on which day the maximum rate of egg-laying for any one day of life was recorded. At day 8, a sharp decline in egg production rate occurred with less than half the standing population laying a single egg. This decline was even more marked the following day, day 9, when a very low rate of egg production with less than one third of the survivors laying a single egg, was evident. No further eggs were recorded for this group after day 9. The Control reproductive period had begun at day 3 and continued to within a day of the total extinction of the group, day 10. However a marked reduction in egg production rate had been recorded at day 8.


KEY
CONTROLS UNTREATED
HIGH CALCIUM CULTURED ROTIFERS
LOW CALCIUM CULTURED ROTIFERS
ROTIFERS TREATED WITH SODIUM CITRATE
TOTAL NUMBER OF EGGS ÷ TOTAL NUMBER OF SURVIVORS
1·44
1·36
1·28
1·20
1·12
1·04
0·96
0·88
0·80
0·72
0·64
0·56
0·48
0·40
0·32
0·24
0·16
0·08
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS OF LIFE

Histogram 2

The High calcium group began egg-laying at day 3 (the same time as the Control group), when four rotifers each laid an egg. Following this day the rate of egg production increased consistently up till day 6, at approximately the same rate of increase as was noted for the Controls. At day 6, the rate of egg-laying began to decline (two days before a decline in rate was recorded for the Control group), and this decline began slowly, continuing more rapidly after day 7, when a very rapid decline in egg production rate was also noted for the Controls. On day 9 less than one third of the standing population laid a single egg, and this was the last day of recorded egg-laying for the High calcium group. The overall reproductive period had extended over the same period as that of the Controls i.e. from days 3 to 9 inclusive, despite the single day's reduction in mean survival time recorded for the group. However, the decline in egg production rate in High calcium individuals preceded any decline in egg-laying in the Control group by two days, and the level of egg production rate in High calcium survivors at days 8 and 9 was slightly below that of the Control group on the same days.

The Low calcium and Citrate treated groups showed a marked delay in the onset of egg-laying, compared to the other two populations. The Low calcium population did not begin egg-laying until day 4 (a day later than the Control group) when 15 rotifers each laid a single egg. Three eggs only were recorded for the Citrate treated group on this day. After day 4, the Low calcium egg-laying increased consistently at a higher rate than that of the Citrate treated group until day 8, when the highest rate of egg production for any experimental group on any single day was recorded for the Citrate treated rotifers. After day 9 the rate of egg production in the Low calcium group showed a slight but only temporary decrease (no such decrease being recorded for the Citrate group until day 11) thereafter maintaining a consistent rate of egg production until day 13 (the last recorded day of egg-laying for the group), with never less than half the standing population laying a single egg on any day. The decrease in egg production rate at day 11 in the Citrate group was again only temporary. Thereafter a consistent rate of egg-laying was maintained in this group until day 15 (the last day of recorded egg-laying for the group), with over half the number of survivors laying a single egg on any day. The


CONTROLS
LOW CALCIUM
HIGH CALCIUM
VOLUME $\mu^3 \times 10^3$
10
9
8
7
6
5
4
3
2
1
DAYS OF LIFE
1
2
3
4

Graph 5

reproductive period of the Low calcium population extended from day 4 to day 13 (two days before the last mortalities were recorded in this group), while that of the Citrate treated rotifers extended from day 4 to day 15 (also two days before the extinction of the group). Compared with the Control reproductive period of 7 days, it was evident that both the populations that displayed increased longevity also displayed increased reproductive periods, although there was an extra day between the end of the reproductive period and the day both groups became extinct compared with the Controls. Furthermore, the rate of egg production at the close of the reproductive period in Low calcium and Citrate treated populations i.e. the last two days, was significantly higher than was the case for the Control population on the same days.

The growth curves for the Control, High and Low calcium groups, while they showed no difference in the duration of the growth period (approximately three days), did show slight differences in growth rate and absolute size attained, (see Graph 5). During the first day of life all groups showed a similar and significant size increase. The rate of growth more than doubled in the case of the High and Control calcium groups on the second day of life, with a slightly higher growth rate being shown by the High calcium population. The Low calcium group showed a significantly lower growth rate early on this day, however its rate of growth did increase half way through it. On day 3, the slightly higher growth rate maintained by the High calcium group over the Controls was reflected in the slightly larger ultimate size in this group. The Low calcium group, although it maintained a growth rate after half-way through the second day that was comparable to the Control rate, still showed a smaller ultimate size as a result of the lower growth rate at the beginning of the second day. Although the Citrate treated group was not measured on the first and second days of life, measurements carried out on the 3rd and 4th days indicated that the growth period ended simultaneously with the other three groups, and the ultimate size correlated closely with that of the untreated Controls.

PRELIMINARY DISCUSSION

ION RATIO EXPERIMENT

The mean longevity of Low calcium - high potassium, and High calcium - low potassium cultured rotifers differed significantly from that of the Controls, with the Low calcium group showing an increase in life expectancy of 40.2% and the High calcium group showing a decrease in life expectancy of 12.6%. The Low calcium - high potassium culture medium differed from the other Knop's media in respect of its similar levels of calcium and magnesium (though the calcium level was lowest of all media and the magnesium level highest), its high potassium level, and its higher ratio between the levels of potassium and magnesium. The question of which of these features, or combination of features, produced life extension is complex until the results of the Citrate treated group are considered, although it may be noted at this stage that in both the culture media that were associated with longevity deviation from the Control group, the level of calcium was inversely proportional to life expectancy, while the levels of magnesium and potassium were directly proportional to life expectancy. The rotifers that had been cultured on Control medium and received treatment with sodium citrate on alternate days of life showed an increased life expectancy of 51.7%. This result would undoubtedly favour an inverse relationship between the level of an ion species and life expectancy in the interpretation of experimental results, since sodium citrate (used here as the tri-sodium salt) is a strong chelating agent possessing 3 electronegative ligand groups per molecule that are capable of specifically withdrawing divalent cations from animal tissues (Lansing and Scott, 1942). As both magnesium and calcium are divalent ion species, and were each present at the same level in the Low calcium medium that was associated with increased longevity, some doubt arises as to whether the withdrawal of both or only one of these ions by chelation was responsible for increased life expectancy.

In relation to this question it should be noted that a low level of magnesium in the High calcium medium did not result in increased life expectancy, but quite the reverse a 12.2% life reduction. While even more important is a consideration of the first order log b values of the formation constants of the complexes formed between calcium and sodium citrate, and magnesium and sodium citrate since these indicate that the calcium/citrate complex is the more likely to be formed (see Table 7). To discover whether the withdrawal of a single ion species such as calcium produced life extension, the chelation experiments were first repeated with chelating agents such as E.G.T.A. and E.D.T.A. which bind calcium 10^6 X and 10^2 X more readily than magnesium.

It is important to note that the rate of egg production in each of the four experimental populations showed good correlation with regard to egg production maxima and length of reproductive period in relation to total longevity. It has been argued by Lansing (1942), et al, (see Page 12) that life extension produced by experimental procedures that merely lower metabolic level, cannot be interpreted as delayed ageing in its strictest sense, and these authors have employed level of egg-laying as an index of metabolic level. On the basis of this sort of assumption, the populations which demonstrated increased life expectancy in the experiments described also showed an increase in total egg production, accompanied on many days of life by an increased rate of egg production over that of the Controls. On the basis of the arguments advanced by Lansing in 1942, this would indicate that delayed ageing in its strictest sense occurred in each of these groups.

MATERIALS AND METHODS

CHELATION EXPERIMENT

Four groups of 30 rotifers were cultured individually on Knop's Control medium throughout life. The first three groups were subjected to 45 second immersions in 0.04 ml volumes of a 0.25% solution of one of the following chelating agents on alternate days throughout life

starting at day 3, E.G.T.A., E.D.T.A., and sodium tartrate. Each rotifer in the group was individually washed in a separate volume of chelating agent after which it was rinsed twice in 0.04 ml volumes of Knop's Control medium before being returned to fresh Control culture. All individual droplets of culture medium were changed daily throughout the duration of experiments and all culture parameters such as food level etc were identical to those described for the Control cultures in the first group of experiments.

The fourth group of rotifers was employed as a parallel Control group, and though it received no treatment with chelating agents its members were washed individually in three separate 0.04 ml volumes of Control medium on the same days (within the extent of its life period) as the other three groups.

Throughout the course of all experiments each experimental group was scored daily for the number of survivors and the number of eggs produced. From the experimental data were plotted a survival graph (Graph 6), and a histogram (Histogram 3) showing the average daily rate of egg production for each individual.

RESULTS

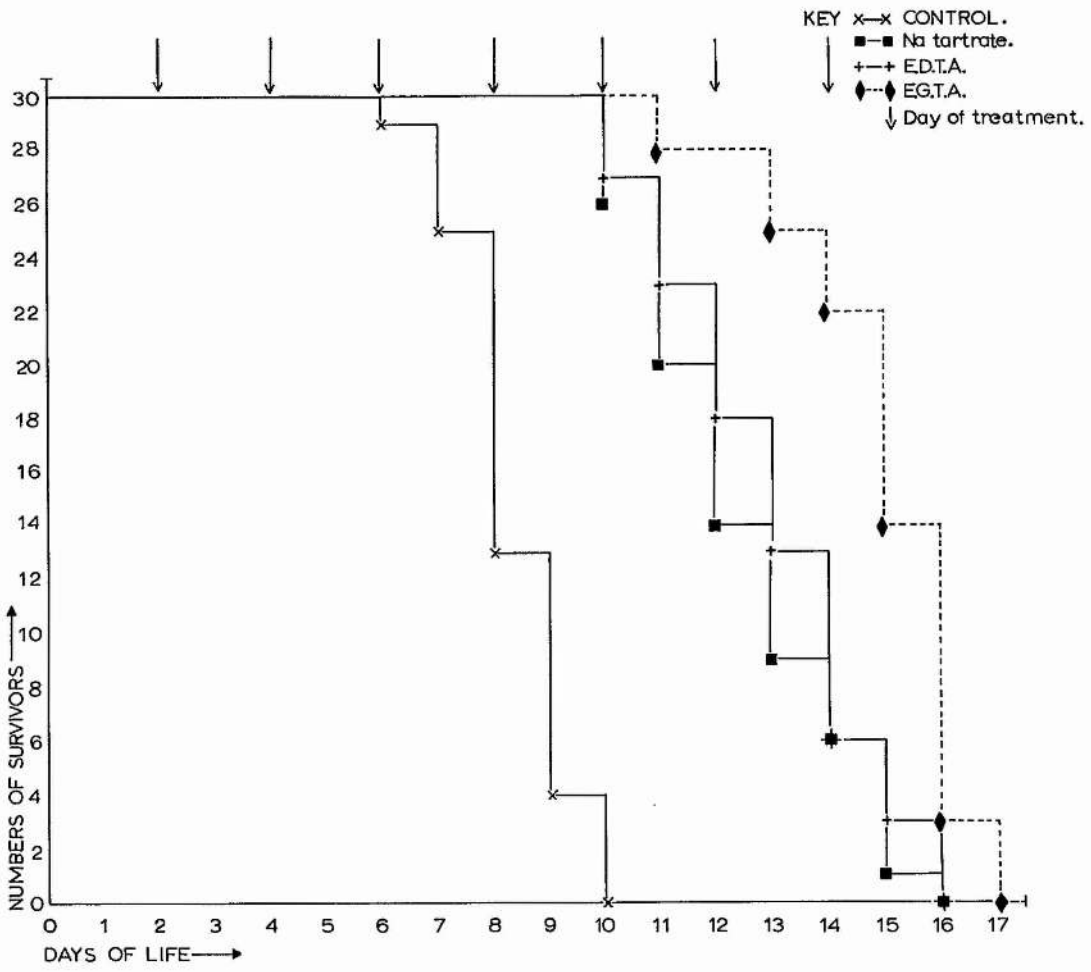
CHELATION EXPERIMENT

The Control survival graph mimics closely the Control graph obtained in the first group of experiments, with the first mortality occurring 2 days later at day 6 (3 days after the period of growth and maturation) (See Graph 6). A particularly sharp decline was again noted at day 8, when 40% of the original Control population died compared with 43% mortality on the same day in the previous graph. The overall mean longevity of 8.7 days was identical to the previously obtained Control mean longevity value, indicating that the additional manipulation and transfers during chelation and washing procedures in this present experiment had no deleterious effect on survival time.

TABLE 6

CHELATION EXPERIMENT

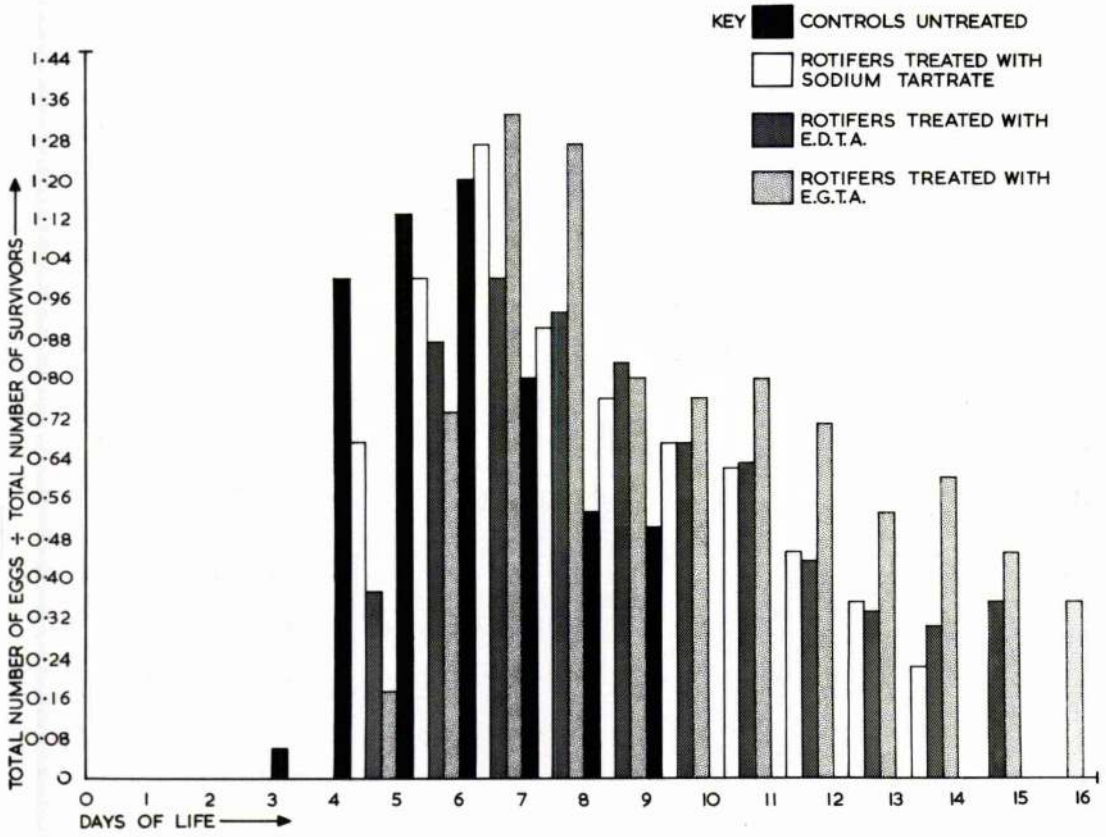
POPULATION	MEAN LONGEVITY	INCREASE IN LIFE EXPECTANCY	TOTAL EGGS LAID
CONTROLS	8.7 days S.E. ± 0.2 days	-	130 eggs
E.D.T.A.	13.0 days S.E. ± 0.3 days	49.4%	202 eggs
Na TARTRATE	12.5 days S.E. ± 0.2 days	43.7%	190 eggs
E.G.T.A.	15.3 days S.E. ± 0.2 days	75.9%	243 eggs


KEY x—x CONTROL.
■—■ Na tartrate.
+—+ E.D.T.A.
♦---♦ E.G.T.A.
↓ Day of treatment.
NUMBERS OF SURVIVORS
DAYS OF LIFE
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Graph 6

The first mortalities in the E.D.T.A. and sodium tartrate treated populations, when three and four rotifers died in each group respectively, occurred at day 10, the day that the last mortalities were recorded for the Control group. The first mortality in the E.G.T.A. group did not occur till the following day, day 11, when two mortalities were recorded. The decline in the sodium tartrate population continued at a slightly greater rate than in the case of the E.D.T.A. group, with a total of three extra mortalities being recorded for the two day period following (days 11 and 12). At day 13 when the same number of deaths were recorded in both these groups, nine rotifers were still alive in the sodium tartrate population compared with 13 survivors in the E.D.T.A. group. The rotifers surviving at this time in the E.G.T.A. group numbered 25, all of the deaths in the group (excluding the first mortalities at day 11) occurring on this day (day 13). On day 14 when there were only six survivors in the sodium tartrate and E.D.T.A. populations, 22 survivors corresponding to 73% of the original population number were recorded in the E.G.T.A. group. However a sharp decline in the number of survivors occurred in this group in the following two days, days 15 and 16 (8 and 11 rotifers dying on each day respectively), such that on day 16 when the last deaths were recorded in the sodium tartrate and E.D.T.A. groups only three survivors were recorded in the E.G.T.A. group, and all of these died the following day, day 17. The mean longevities of the sodium tartrate and E.D.T.A. treated groups were 12.5 and 13.0 days corresponding to an increase in life expectancy over the Control group of 43.7% and 49.4% respectively (see Table 6). The E.G.T.A. treated rotifers showed a mean longevity of 15.3 days, and this corresponded to an increased life expectancy of 75.9%.

The reproductive period in the Control group began at day 3 and ended at day 9, as was found in the Control group in the Ion ratio Experiment, (see Histogram 3). A slight decline in rate of egg production occurred at day 7 in this group, one day before a sharp decline was noted in the Controls of the previous experiment, however this earlier decline was more than offset by the higher rate of egg-laying recorded during all other days of the reproductive period except the first.


KEY
CONTROLS UNTREATED
ROTIFERS TREATED WITH SODIUM TARTRATE
ROTIFERS TREATED WITH E.D.T.A.
ROTIFERS TREATED WITH E.G.T.A.
TOTAL NUMBER OF EGGS ÷ TOTAL NUMBER OF SURVIVORS
1·44
1·36
1·28
1·20
1·12
1·04
0·96
0·88
0·80
0·72
0·64
0·56
0·48
0·40
0·32
0·24
0·16
0·08
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS OF LIFE

Histogram 3

The onset of egg-laying was delayed by one day till day 4 in all the experimental groups that received treatment with a chelating agent, with a particularly marked delay occurring in the E.G.T.A. group which only laid five eggs on this day. Following the delay in the start of the reproductive period, all treated groups showed a consistent and rapid increase in egg production rate at day 5, although slightly less than three quarters of the E.G.T.A. population laid an egg on this day. At day 6 all groups showed their highest rate of reproduction during their reproductive period, with the rate of egg-laying in the E.G.T.A. population showing a sharp increase from day 5 to the highest level of egg-laying recorded for any single population during the entire course of the experiment. This high rate of egg production was continued in the E.G.T.A. group on the following day, day 7, the same day that the Control and sodium tartrate treated groups both showed declines in their rates of egg production. The E.G.T.A. group did not show a significant decline in rate until the following day, day 8, when rate of egg-laying fell to approximately the same level as that shown by the other treated groups, a level that was significantly higher than the Control level of egg-laying recorded on the same day. After the last day of Control egg-laying at day 9, the sodium tartrate and E.D.T.A. groups showed a similar decline in their egg-laying up to days 13 and 14 respectively, the last days of recorded egg production for each group. From day 9 the E.G.T.A. population had shown a significantly higher average rate of egg-laying than in any of the other groups, indeed as late as day 14, five days after the last day of Control egg-laying, over half the surviving population laid a single egg. The final day of egg production recorded for the E.G.T.A. group was day 16.

The reproductive period in all populations that received treatment with a chelating agent showed considerable extension over that of the Controls. In the E.G.T.A. group the period of reproduction extended over 13 days (six days longer than the corresponding period in the Control group), while the reproductive period in sodium tartrate and E.D.T.A. treated populations extended over 11 and 12 days respectively (four and five days longer in each case than the corresponding Control period).

TABLE 7

Chelating Agent	1st order log B value of formation constant with divalent ions.		Increased specificity for Ca.	Increase in life expectancy
	Mg	Ca		
E.G.T.A.	5.20	10.97	10^6 X	75.9%
E.D.T.A.	8.69	10.59	10^2 X	49.4%
(Na citrate	[x] 3.16	[x] 3.55	2.5 X	51.7%)
Na tartrate	1.91	2.17	1.8 X	43.7%

x The log B values of this chelating agent correspond to the approximately doubled ionic strength at which it was employed (see Ion Ration Experiments), the Na citrate data being included here for convenience rather than strictly belonging to the series.

PRELIMINARY DISCUSSION

CHELATION EXPERIMENT

The log b value of the formation constant may be taken as an index of the affinity of a chelating agent for a particular ion species. The highest log b value for a complex between calcium and any of the chelating agents used is evident for the complex formed between calcium and E.G.T.A. (see Table 7). Furthermore since the corresponding log b value for a complex involving magnesium and the same chelating agent is about 10^6 X lower than for a complex formed with calcium, E.G.T.A. may be said to exhibit a 10^6 X greater calcium specificity, which value is in fact about 10^4 X higher than the second most calcium specific chelating agent used in this experiment - E.D.T.A. In this context it is particularly significant that the greatest increase in life expectancy recorded in any experimental group was found in the population treated regularly throughout life with E.G.T.A. Furthermore, the E.G.T.A. survival graph together with the survival graph of the Controls showed a similar closer approximation to a rectangular form than was the case in any of the other groups, with few early mortalities coupled with a sharp decline in the number of survivors at the end of life, both features of a healthy senescing population well adjusted to its conditions of culture. E.D.T.A. shows a log b value of formation constant with calcium 100 X greater than the corresponding value for magnesium, indicating a 100 fold increase in calcium specificity, while sodium tartrate shows a much lower specificity for calcium with a much lower value of formation constant. In this context it is interesting to note that the increase in life expectancies in both the E.D.T.A. and sodium tartrate treated populations was 49.4% and 43.7% respectively - both very similar values. A possible explanation for this result may have been that E.D.T.A. was comparatively more toxic to the species than sodium tartrate, indeed, it can be readily demonstrated that a single 45 second immersion of a 5 day old rotifer in a 0.04 ml volume of a 0.5% solution of sodium tartrate produces no immediate harmful effects, while immersion of a 5 day old in a similar volume of 0.5% E.D.T.A. produces instant mortality.

No evidence was found in the rates of egg-laying of the groups that showed an increase in life expectancy of a lowered metabolic rate (see Lansing's argument cited on Page 12), indeed a significant rate of egg laying continued to within one, two and three days of the final mortalities in the E.G.T.A., E.D.T.A., and sodium tartrate treated groups respectively. Again evidence was found in the populations that displayed life extension of a single day's delay in the onset of egg-laying, particularly in the case of the E.G.T.A. population that also featured the highest life expectancy of all groups.

In order to discover whether or not calcium was accumulated throughout life in this species, and whether or not this accumulated calcium was removed by treatment with chelating agents, a series of radiotracer studies were carried out employing the radionuclide 45 calcium and the technique of scintillation counting.

MATERIALS AND METHODS

RADIOTRACER EXPERIMENT 1.

Three groups of individually cultured rotifers were maintained in one of the 3 modified Knop's media described in the Ion Ratio Experiment. The radionuclide 45 calcium had been added to each medium in a quantity that preserved the ratios of total calcium concentration between media at the original 3:2:1 ratio. The total salt concentration in each medium was carefully maintained at 0.04%. Otherwise the individual cultures were maintained exactly as described in the Ion Ratio Experiment Page 36 , with the notable exception that all culture droplets were established on plastic and not glass slides in order to prevent exchange occurring between the 40 calcium silicates of glass and the 45 calcium of the radioactive culture media, which would have produced variabilities in the specific activity of the culture medium in each droplet. On every day of life 10 individuals were removed from each of the three radioactive culture sets and washed individually in three separate 3 ml washing volumes, consisting of the appropriate 40 calcium Knop's medium in which the animals had been cultured. All rotifers were transferred after washing to separate

filter discs where they were stored till the end of the experiment, when all samples were counted by scintillation counting technique on the same day to avoid having to make individual corrections for decay factors.

Two additional groups of individually cultured rotifers were maintained on Control medium supplemented with 45 calcium. One of these groups was subjected to individual 45 second immersions in separate 0.04 ml volumes of 0.5% sodium citrate on alternate days in exactly the same manner as described for the Ion Ratio Experiment, with the important difference that ten rotifers in the group were washed in three separate 3 ml volumes of Control 40 calcium Knop's medium before chelation, and the washings of these ten rotifers transferred to ten separate filter discs (renewed on each day of treatment), to be pooled till the end of the experiment and counted by scintillation counting technique on the same day as all other experimental samples. The other additional group of individually cultured rotifers received treatment with sodium citrate on alternate days again exactly as described in the Ion Ratio Experiment, with the important difference that ten rotifers out of the population were washed in three separate 3 ml volumes of Control 40 calcium Knop's medium after receiving chelation treatment, and these ten rotifers were then transferred to ten separate filter discs (renewed on each day of treatment), to be counted by scintillation counting technique, on the same day as other experimental samples. In this way two sets of experimental data were obtained, one of the sodium citrate washings of rotifers that had been subjected to brief immersions in 0.04 ml volumes of a solution of this chelating agent on alternate days of life, and the other of rotifers that had been cultured on the same radioactive medium and had also been subjected to brief immersion in sodium citrate solution, on the same days of life as the other group, before sampling.

RESULTS

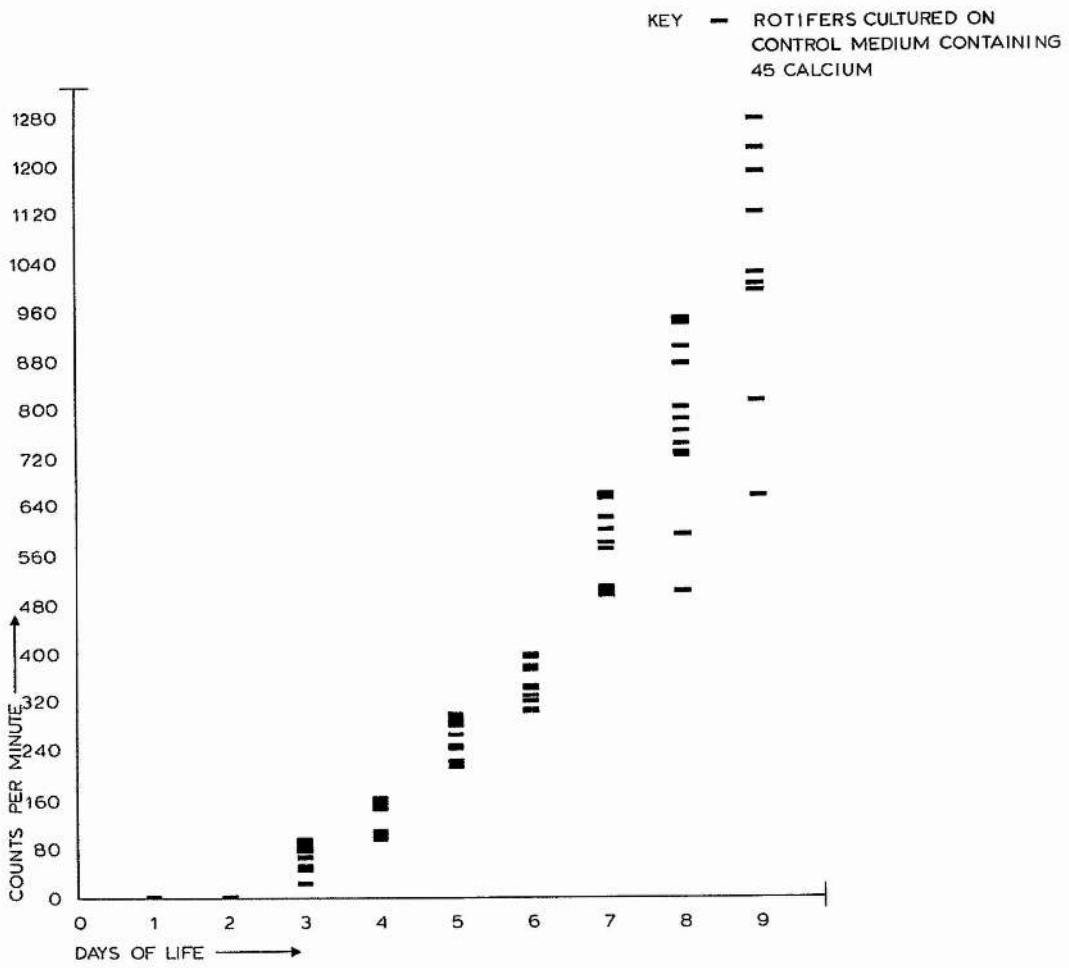
RADIOTRACER EXPERIMENT 1

Continued exposure to 45 calcium in Control radioactive medium, showed an average accumulation of 45 calcium that began on day 3 (at

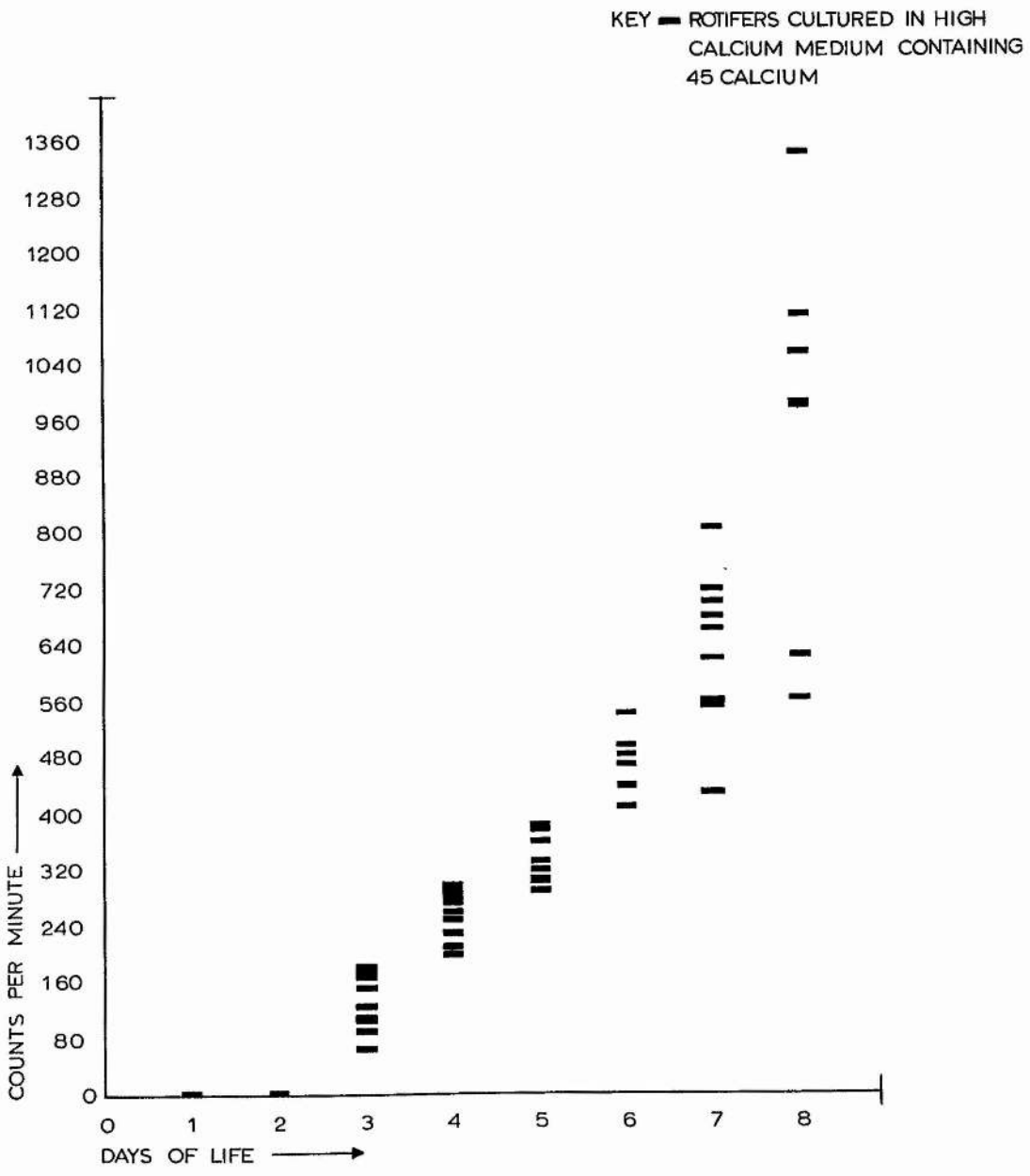
TABLE 8

RADIOTRACER EXPERIMENT 1

POPULATION	MEAN COUNTS PER MINUTE/DAY OF LIFE Below corresponding weights X 10^{-12} gms														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CONTROLS			56 4.59	156 12.75	272 22.43	330 27.27	539 44.13	774 63.39	1060 86.82						
HIGH			132 10.80	248 20.10	349 28.59	466 48.16	650 53.22	1043 85.41							
LOW			32 2.61	57 4.68	69 5.64	83 6.78	112 9.18	153 12.54	218 17.85	318 25.74	666 54.54	780 63.87	814 66.66		
CITRATE ROTIFERS					60 4.91		100 8.19		198 16.21		300 24.57		508 41.61		782 61.05
CITRATE WASHINGS			57 4.67		94 7.68		128 10.48		140 11.17		200 16.38		315 25.50		405 33.17
% AGE CALCIUM REMOVED			100		61		56		41		40		38		34


KEY — ROTIFERS CULTURED ON CONTROL MEDIUM CONTAINING 45 CALCIUM
1280
1200
1120
1040
960
880
800
720
640
560
480
400
320
240
160
80
0
COUNTS PER MINUTE
0
1
2
3
4
5
6
7
8
9
DAYS OF LIFE

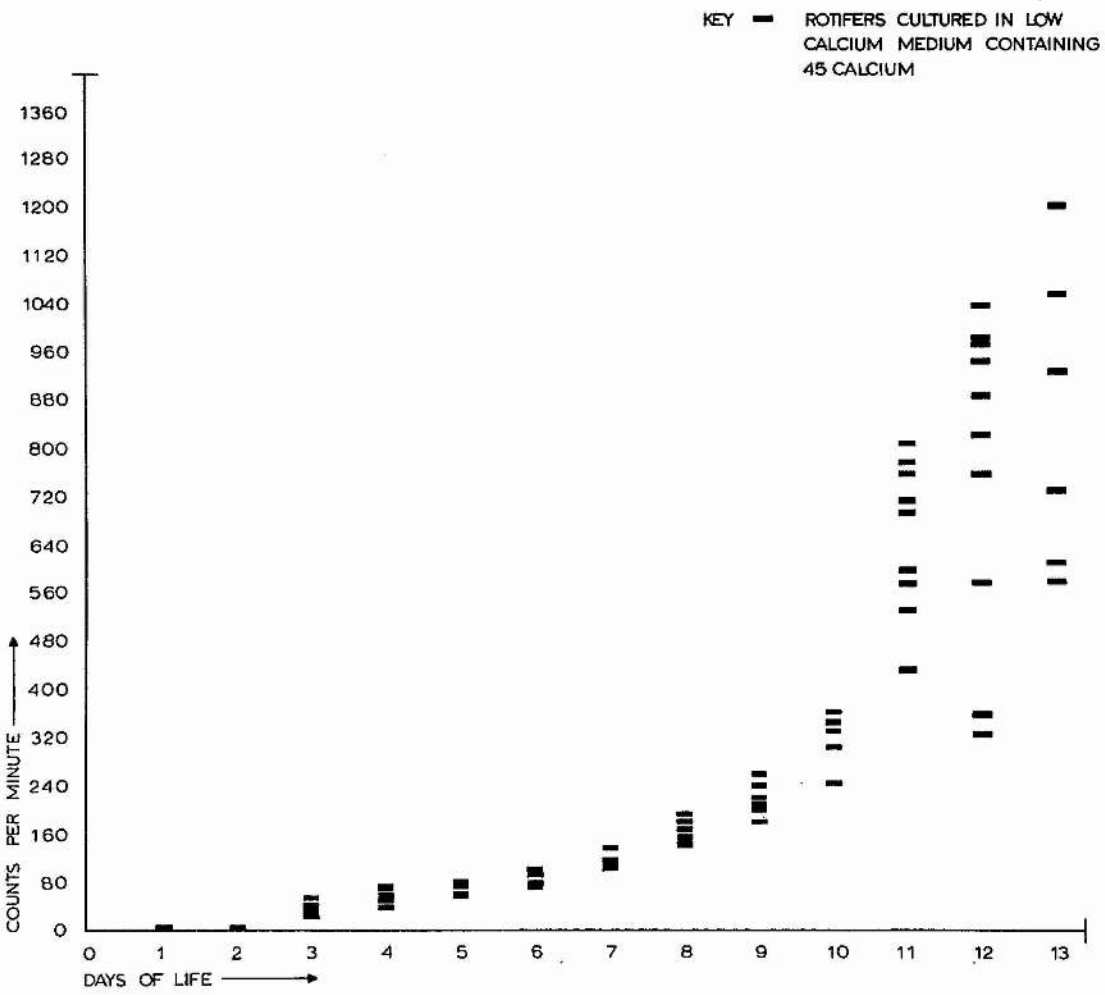
Graph 7


KEY — ROTIFERS CULTURED IN HIGH CALCIUM MEDIUM CONTAINING 45 CALCIUM
COUNTS PER MINUTE
1360
1280
1200
1120
1040
960
880
800
720
640
560
480
400
320
240
160
80
0
0
1
2
3
4
5
6
7
8
DAYS OF LIFE

Graph 8

the end of the period of growth and maturation), when a low average count of 56 cpm (4.59×10^{-12} gms) was obtained (see Graph 7 and Table 8). The total average 45 calcium count at day 4 was 100 cpm above the previous day's value, corresponding to a 2.8X increase in the rate of 45 calcium accumulation on this day. No significant alteration in this daily rate of 45 calcium accumulation was noted till day 6, when the average 45 calcium intake rate declined to almost the same value as that initially shown at day 3. However, the total 45 calcium count of samples increased on days 7, 8 and 9, by 3.6X, 4X and 5X, respectively, the rate of 45 calcium accumulation shown at day 6. The final total of 45 calcium accumulated in Control samples at day 9, corresponded to 1060 cpm (86.82×10^{-12} gms).

Continued exposure to 45 calcium in High calcium radioactive medium showed an accumulation of 45 calcium that began on the same day as the Control samples, on day 3, at 2.4 X the Control rate (see Graph and Table 8). On days 4 and 5 the High calcium samples showed an average uniform rate of 45 calcium accumulation of about 100 cpm/day, corresponding to the same accumulation rate as the Control samples on these days, however the average total count for the High calcium samples was equivalent to 84 cpm/day above that of the Control samples on each day, as a result of the initially more than doubled rate of 45 calcium intake at day 3. The decline in the average rate of 45 calcium accumulation noted in the Control samples at day 6, was not paralleled by a similar decline in the High calcium samples counted on this day, which showed a continued accumulation of 45 calcium at about the same rate (100 cpm/day) as on the previous 3 days. At day 6 the average total count for the High calcium samples was 136 cpm higher than the average Control count on the same day, corresponding to an increased weight of 45 calcium accumulated of 20.9×10^{-12} gms. The High calcium samples counted at day 7, showed an increased average 45 calcium accumulation rate that corresponded to 1.6 X the rate of the previous day, day 6, while at day 8 a further increase in accumulation rate corresponding to 2.1 X the day 7 value was observed, and this reflected a total 45 calcium accumulation that was the highest for any single day during

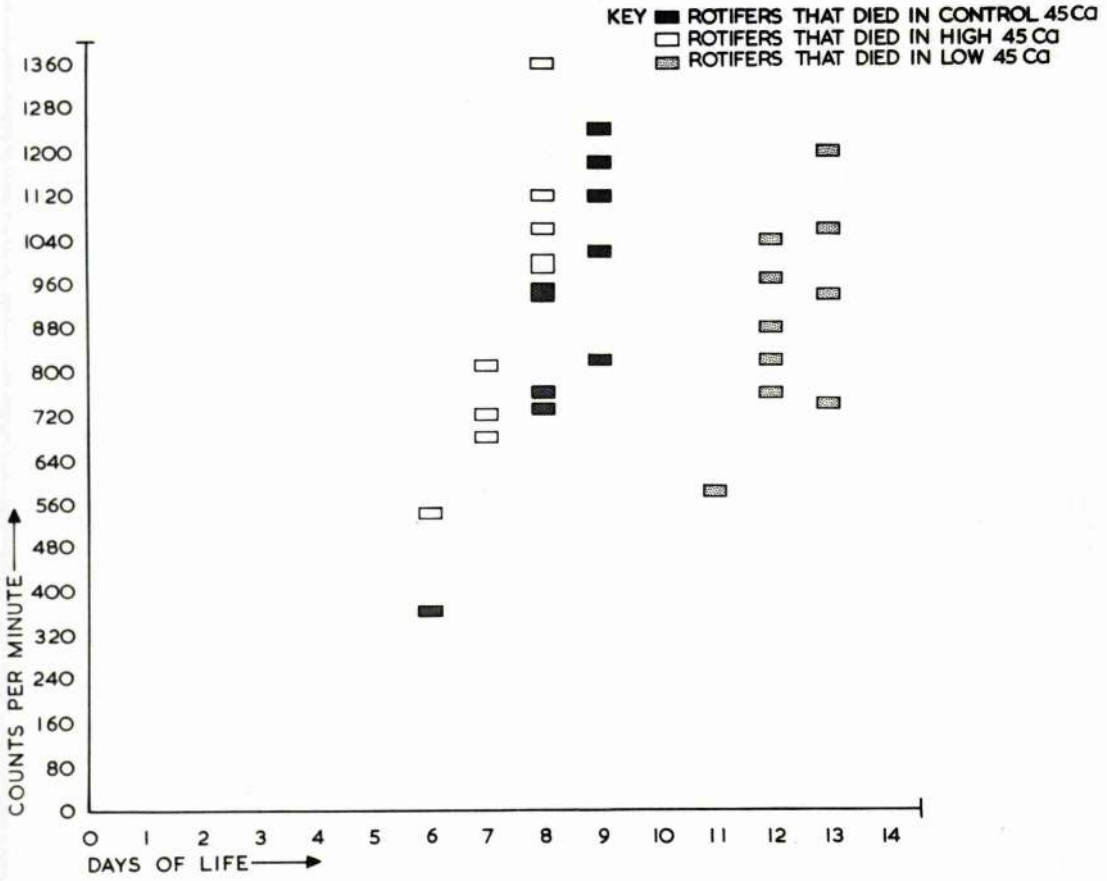

KEY
ROTIFERS CULTURED IN LOW CALCIUM MEDIUM CONTAINING 45 CALCIUM
COUNTS PER MINUTE
1360
1280
1200
1120
1040
960
880
800
720
640
560
480
400
320
240
160
80
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
DAYS OF LIFE

Graph 9

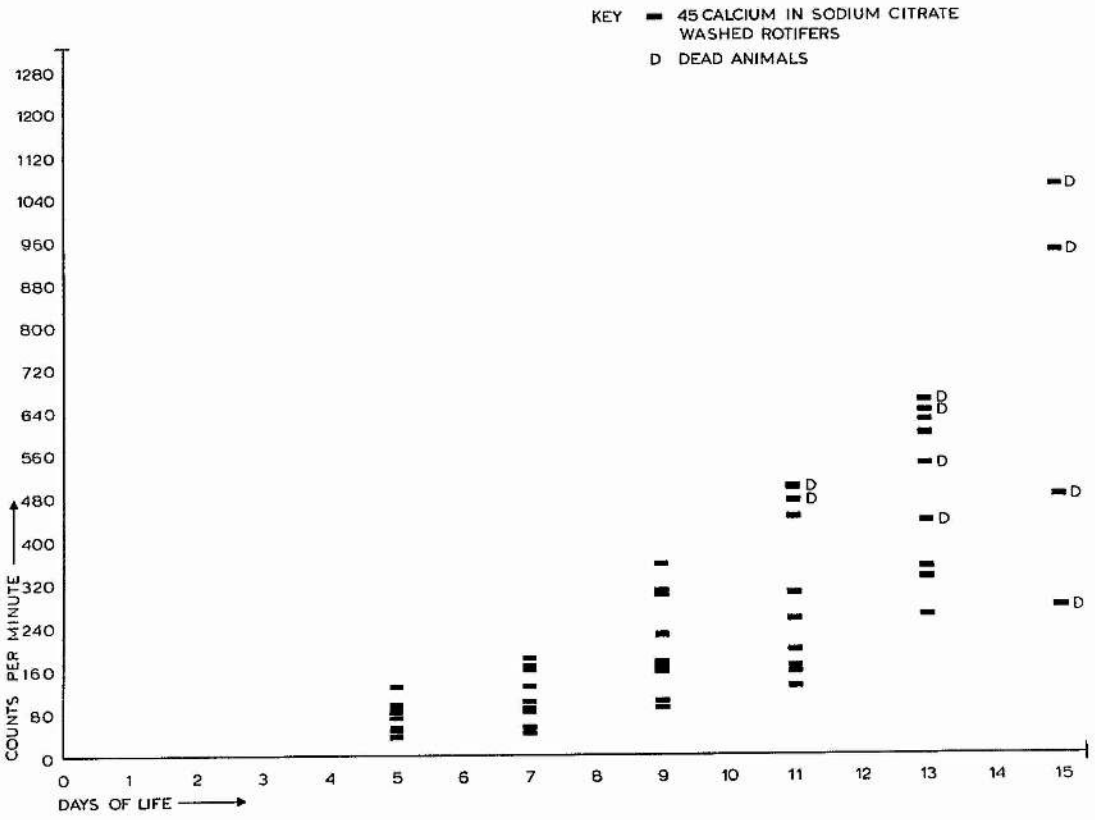
the life period. The Control samples also showed increases in total 45 calcium content at days 7 and 8, but whereas the accumulation at day 7 was approximately equivalent to that in the High calcium samples on the same day, at day 8 it was 1.6 X lower than the average count of the corresponded day of the High calcium rotifers. The final count in the High calcium samples at day 8, corresponded to an average total count of 1043 cpm (85.41×10^{-12} gms), and was comparable to the final count of 1060 cpm (86.82×10^{-12} gms) obtained for the final Control samples the following day.

The first accumulation of 45 calcium in the rotifers cultured in Low calcium radioactive medium occurred at day 3 (at the end of the period of growth and maturation), when just over half the number of counts registered for the Control samples on this day were recorded (see Graph 9 and Table 8). Thereafter, the average rate of 45 calcium accumulation per day till day 9 in the Low calcium samples was as low as 31 cpm per day, with the highest day increase of 65 cpm being recorded at day 9, when the total average count of 218 cpm was 832 cpm (68.13×10^{-12} gms) lower than the average final count of the Controls on the same day. Following day 9 the Low calcium samples showed increased rates of 45 calcium accumulation at each day up till day 11, when the average total count of 348 cpm recorded on this day represented the highest 45 calcium accumulation rate on any single day during the entire life period. The count obtained from the Low calcium samples at day 12 showed an accumulation of 45 calcium that had declined to one third of the rate of the previous day, and this decline in accumulation rate continued at day 13, when a final count of 814 cpm (20.16×10^{-12} gms or 246 cpm below the final Control values) reflected an accumulation rate that was approximately equivalent to that exhibited during the early part of life - up till day 9.

The counts obtained from rotifers that died on the same day that they were sampled in each of the groups cultured on the three modified radioactive Knop's media were recorded separately (see Graph 10).


KEY ROTIFERS THAT DIED IN CONTROL 45 Ca
ROTIFERS THAT DIED IN HIGH 45 Ca
ROTIFERS THAT DIED IN LOW 45 Ca
COUNTS PER MINUTE
1360
1280
1200
1120
1040
960
880
800
720
640
560
480
400
320
240
160
80
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS OF LIFE

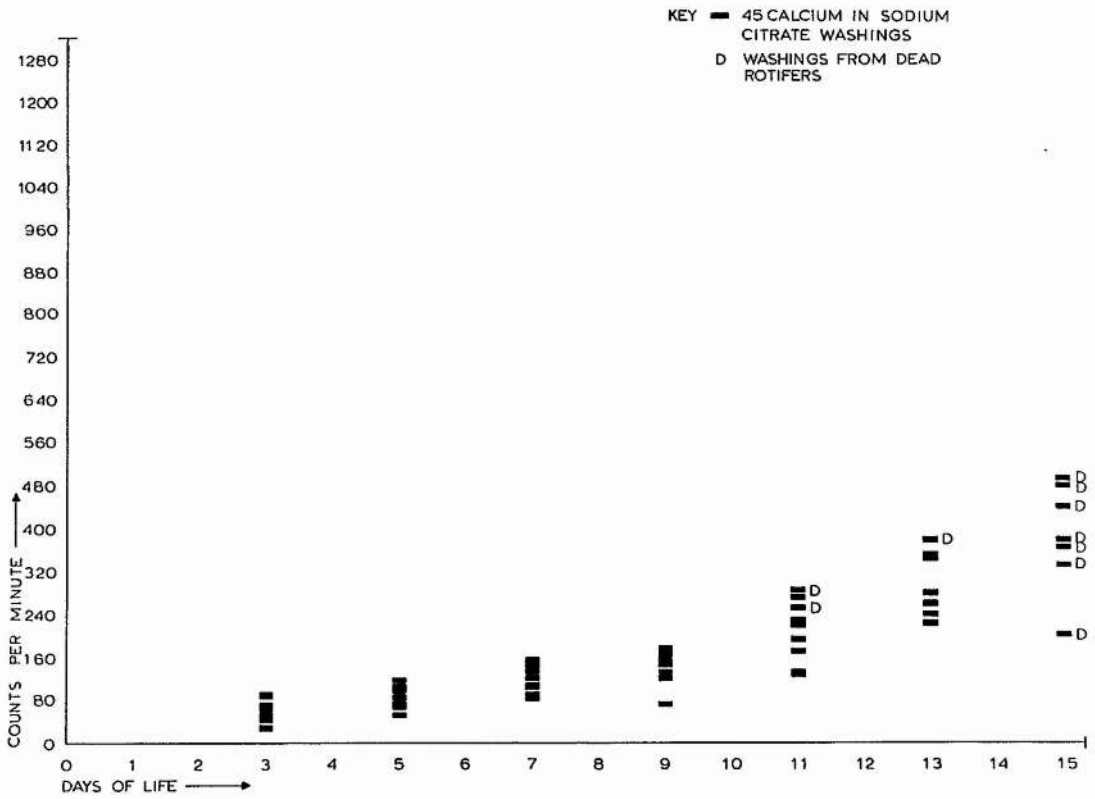
Graph IO


KEY
45 CALCIUM IN SODIUM CITRATE WASHED ROTIFERS
D DEAD ANIMALS
COUNTS PER MINUTE
1280
1200
1120
1040
960
880
800
720
640
560
480
400
320
240
160
80
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
DAYS OF LIFE

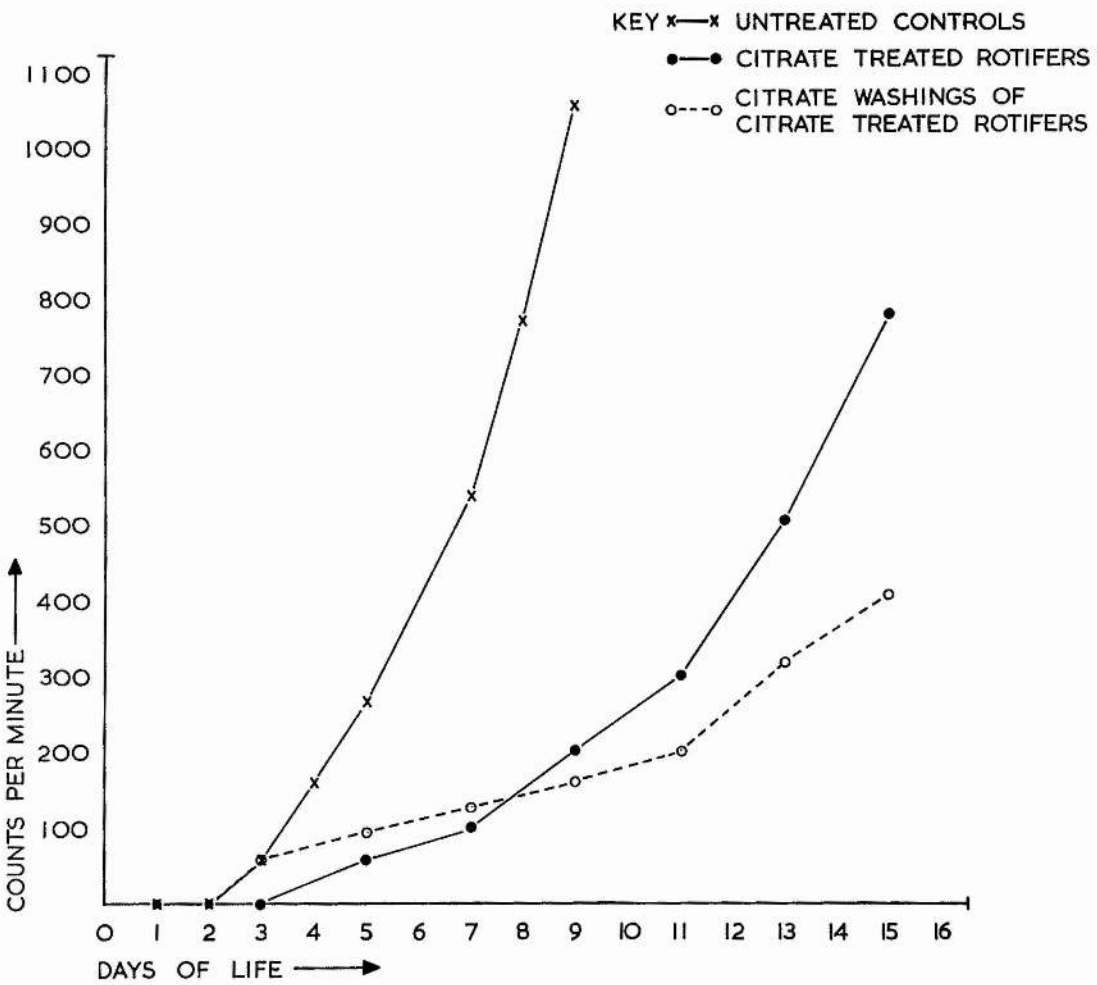
Graph 11

Only 3 dead rotifers produced a count below 640 cpm from any of the three sets, indicating that regardless of the Knop's medium on which rotifers had been cultured during life a considerable accumulation of 45 calcium was evident at the end of it.

Rotifers that were cultured on 45 calcium Knop's Control medium and received treatment with sodium citrate, began taking in 45 calcium in detectable amounts between days 3 and 5, with no counts being recorded at day 3 when the first counts were noted for the untreated Controls (see Graph 11 and Table 8). The total 45 calcium intake at day 5 in the citrate treated rotifers corresponded to 60 cpm, 212 cpm below the total count recorded for the untreated Control rotifers, and only 9 cpm below those recorded for the Low calcium samples on this day. At day 7 the citrate treated rotifers had only accumulated a further 40 cpm from day 5, compared with a similar accumulation over the same period in the Low calcium samples, and a much higher accumulation of 267 cpm in the untreated Controls. On day 9 when the final count of 1060 cpm (86.82×10^{-12} gms) was recorded for the untreated Controls, the citrate treated rotifers registered a total 45 calcium count of 198 cpm (16.21 gms), 862 cpm (70.61×10^{-12} gms) below the final count of the Untreated Controls and only 20 cpm (1.64×10^{-12} gms) below a total count obtained for Low calcium samples on the same day. The average rate of 45 calcium accumulation for the citrate treated Controls between days 7 and 9 was approximately 2.2 X the average accumulation rate between days 5 and 7, and corresponded to about 50 cpm per day. This average rate of 45 calcium accumulation continued unaltered till day 11, when the total 45 calcium count obtained for the citrate treated rotifers was 300 cpm (24.57×10^{-12} gms), 366 cpm (29.97×10^{-12} gms) below the corresponding day count of the Low calcium samples, which had shown a marked increase in their rate of 45 calcium accumulation on this day. From days 11 to 13 the average 45 calcium intake in the citrate treated rotifers occurred at double the average rate of 50 cpm per day observed during the previous 4 days (days 7 to 11), giving a total 45 calcium count at day 13 of 508 cpm (41.61×10^{-12} gms), 306 cpm (25.06×10^{-12} gms) below the final count obtained for the Low calcium samples on the same day. The average rate of 45 calcium intake in the citrate treated rotifers between days 13 and 15 rose slightly to 1.3 X the intake rate of the


KEY 45 CALCIUM IN SODIUM CITRATE WASHINGS
D WASHINGS FROM DEAD ROTIFERS
COUNTS PER MINUTE
1280
1200
1120
1040
960
880
800
720
640
560
480
400
320
240
160
80
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
DAYS OF LIFE

Graph 12


KEY ×—× UNTREATED CONTROLS
●—● CITRATE TREATED ROTIFERS
o---o CITRATE WASHINGS OF CITRATE TREATED ROTIFERS
COUNTS PER MINUTE
1100
1000
900
800
700
600
500
400
300
200
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS OF LIFE

Graph 13

previous two day period, producing a final total 45 calcium count in this set of 782 cpm (61.05 X 10^{-12}gms), 278 cpm (22.77 X 10^{-12}gms) below the final count obtained for the untreated Controls at day 9, and 24 cpm (1.96 X 10^{-12}gms) below the final count obtained for the Low calcium samples at day 13.

The counts obtained for the sodium citrate washings of rotifers cultured on 45 calcium Knop's Control medium throughout life, and subjected to treatment with sodium citrate on alternate days, indicated a withdrawal of 45 calcium (corresponding to 57 cpm, 2.61 X 10^{-12}gms) which occurred at day 3 on the first day of washing, when no total 45 calcium count was obtained for the citrate treated rotifers and a similar accumulation of 45 calcium (56 cpm) was obtained for the untreated Control samples (see Graphs 12 and 13). The total 45 calcium withdrawn from rotifers by sodium citrate on this day as a percentage of the total 45 calcium present in rotifers before the washing treatment, corresponded to 100% withdrawal, (see Graph 14). At day 5 when the first total 45 calcium counts of 60 cpm were obtained for the citrate treated rotifers, the 45 calcium present in sodium citrate washings had only increased by 37 cpm from the counts obtained at day 3, this producing a 39% reduction in the total 45 calcium withdrawn as a percentage of the total 45 calcium present before citrate treatment, the greatest reduction in the percentage of 45 calcium withdrawn for any day of treatment during the life period. At day 7 a similar 45 calcium increase of 34 cpm was noted in the sodium citrate washings, however this slight increase produced little reduction in the percentage of 45 calcium withdrawn as a similar increase of 40 cpm was recorded for the citrate treated Controls on this day. On each day of citrate treatment up till day 9 a higher 45 calcium count was obtained for the citrate washing medium than the citrate treated rotifers. On this day however, the total 45 calcium count recorded for the washing medium, which had increased by only 12 cpm from day 7, was 58 cpm below that obtained for the citrate treated rotifers which had increased their total 45 calcium count by almost 100 cpm from the previous day of treatment. This almost negligible increase in the total 45 calcium withdrawn into

TABLE 9

DAY	ANIMAL	WASHINGS	TOTAL
3	0	57	57
5	60	94	211
7	100	128	379
9	200	140	619
11	300	200	919
13	508	315	1442
15	782	405	2121
			1339

the citrate medium relative to the increased rate in the total 45 calcium accumulated in the citrate treated rotifers, produced a 14.72% reduction in the percentage 45 calcium withdrawn from rotifers as a fraction of the total 45 calcium present before treatment on this day. At day 11 the citrate washings showed an increase in total 45 calcium content of 60 cpm, however a further slight reduction in the percentage of 45 calcium withdrawn corresponding to 1.42% was noted, as this value was 42 cpm below the total increase in 45 calcium accumulated. On days 13 and 15, the total 45 calcium present in the sodium citrate washings increased by 115 cpm and 90 cpm on each day respectively, however both these values were associated with 1.73% and 4.16% reductions respectively in the percentage of 45 calcium withdrawn, owing to greater increases in the total 45 calcium accumulated being recorded in the citrate treated Control rotifers on each day. The 45 calcium withdrawn in sodium citrate at day 15, the final day of citrate treatment, as a percentage of the total 45 calcium present in Control rotifers before treatment, corresponded to 34.11%, the lowest value obtained on any day of treatment during the entire life period.

When the total counts for the 45 calcium withdrawn by sodium citrate are added to the corresponding day counts obtained for the citrate treated rotifers on each of the days of treatment, it can be seen that the values obtained do not equal the equivalent day values obtained for the untreated Control rotifers after day 3 (see Table 9). Indeed at day 9 when the final 45 calcium counts were obtained for the untreated Control samples, the summed counts of the citrate treated rotifers and sodium citrate washings on this day, are 424 cpm below the total 45 calcium counts in the untreated Controls. This would indicate that quite apart from the 45 calcium removed at each citrate treatment, which totals 1339 cpm or 109.66×10^{-12} gms during the life-time of a citrate Control rotifer, there is a reduction in the rate of 45 calcium accumulation between the days of treatment.

TABLE 10	CONTROL MEDIUM	LOW CALCIUM MEDIUM	HIGH CALCIUM MEDIUM
WT. OF 40 Ca in 0.02 mls.	4.8×10^{-6} gms	2.4×10^{-6} gms	7.2×10^{-6} gms
WT. OF 45 Ca IN 0.02 mls.	0.024×10^{-6} gms	0.012×10^{-6} gms	0.036×10^{-6} gms
RATIO OF 40 Ca to 45 Ca	200 to 1	200 to 1	200 to 1
TOTAL MASSES OF Ca TAKEN UP BY ROTIFERS ON EACH DAY OF LIFE	DAY 1 DAY 2 DAY 3 0.92×10^{-9} gms DAY 4 2.55×10^{-9} gms DAY 5 4.49×10^{-9} gms DAY 6 5.45×10^{-9} gms DAY 7 8.83×10^{-9} gms DAY 8 12.68×10^{-9} gms DAY 9 17.36×10^{-9} gms	DAY 1 DAY 2 DAY 3 0.32×10^{-9} gms DAY 4 0.94×10^{-9} gms DAY 5 1.13×10^{-9} gms DAY 6 1.36×10^{-9} gms DAY 7 1.84×10^{-9} gms DAY 8 2.51×10^{-9} gms DAY 9 3.57×10^{-9} gms DAY 10 5.15×10^{-9} gms DAY 11 10.91×10^{-9} gms DAY 12 12.77×10^{-9} gms DAY 13 13.33×10^{-9} gms	DAY 1 DAY 2 DAY 3 2.16×10^{-9} gms DAY 4 4.02×10^{-9} gms DAY 5 5.72×10^{-9} gms DAY 6 9.63×10^{-9} gms DAY 7 10.64×10^{-9} gms DAY 8 17.08×10^{-9} gms
TOTAL MASSES OF Ca TAKEN UP BY CITRATE TREATED ROTIFERS	DAY 3 DAY 5 0.98×10^{-9} gms DAY 7 1.64×10^{-9} gms DAY 9 3.24×10^{-9} gms DAY 11 4.91×10^{-9} gms DAY 13 8.32×10^{-9} gms DAY 15 12.21×10^{-9} gms		
TOTAL MASSES OF Ca PRESENT IN THE CITRATE WASHINGS OF CITRATE TREATED ROTIFERS	DAY 3 0.93×10^{-9} gms DAY 5 1.54×10^{-9} gms DAY 7 2.10×10^{-9} gms DAY 9 2.23×10^{-9} gms DAY 11 3.28×10^{-9} gms DAY 13 5.10×10^{-9} gms DAY 15 6.63×10^{-9} gms		
TOTAL 45 Ca ACCUMULATED AT THE LAST DAY OF LIFE AS A FRACTION OF THE TOTAL 45 Ca PRESENT IN 0.02 ml OF MEDIUM	0.36% (0.25% for citrate treated rotifers)	0.55%	0.24%

PRELIMINARY DISCUSSION

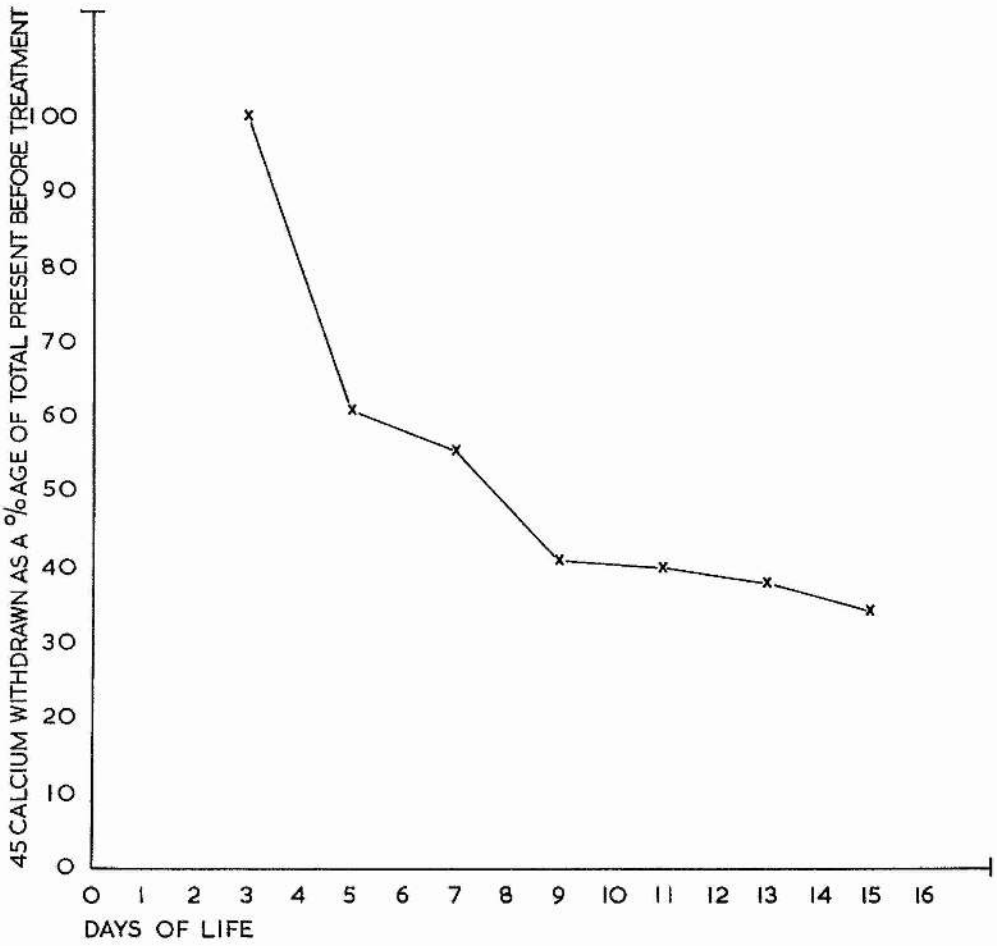
RADIOTRACER EXPERIMENT 1

It is important to remember while discussing the results of the radioactive experiments performed in this study, that the radionuclide level in each of the three modified Knop's media represented 1/200th of the total 40 calcium level in each 0.02 ml culture droplet. All absolute mass estimates are thus multiplied by a factor of 200, (see Table 10), since there was no evidence to contradict the assumption that the inactive and active nuclides were equivalent. However, it must be emphasised that the purpose of these radiotracer experiments was to trace comparatively the general patterns of calcium intake in each of the groups of rotifers during life, rather than estimating the absolute masses of calcium accumulated.

The total 45 calcium counts obtained for the rotifers sampled from Control, Low and High calcium radioactive media on each day of life, showed significant increases in 45 calcium content that began at the end of the period of growth and maturation and continued in the manner of an accumulation throughout the entire life period. The average daily rate of 45 calcium accumulation during the period of sampling in the 3 groups was inversely related to the total mean longevity in each group, such that compared to the Control rate of accumulation of 118 cpm per day the slightly higher daily rate of accumulation of 130 cpm in the High calcium group was associated with a slight reduction in mean longevity of 12.6%, while the average daily intake rate of 63 cpm in the Low calcium group, which was about half the rate of intake in the Controls, was associated with a 40.2% increase in life-span. All populations showed their highest rates of 45 calcium accumulation during the last few days of sampling however, whereas the Control and High calcium cultured samples showed their highest 45 calcium intakes on the final day of counting, the final count obtained for the Low calcium samples did not reflect the high increases of the previous 3 days. The total 45 calcium

counts obtained for rotifers that died in their respective culture media on the day they were sampled, were above 640 cpm with only three exceptions in the populations of the 3 sets. It is interesting to note that the total weight of 45 calcium accumulated as a fraction of the total weight of 45 calcium present in the culture medium differed in each of the 3 experimental populations, (see Table 10). In order to produce a final count of 800 cpm (only 200 cpm below that of the Controls) the Low calcium rotifers withdrew the highest fraction of 45 calcium from their medium over the longest period of time, while in the case of the Control samples a high final count (1060 cpm) produced over a shorter period of exposure was more than compensated by the increased 45 calcium content of the Control radioactive medium. The High calcium samples over a still shorter exposure period withdrew a low fraction of 45 calcium (0.12% below that of the Controls), and this was a reflection of the similarity between the final High calcium count of 1043 cpm and that of the final count of the Controls, 1060 cpm.

The rotifers that were cultured in Control 45 calcium medium and received treatment with sodium citrate on alternate days, showed an increase in 45 calcium content that began after day 3 and continued in the manner of an accumulation throughout life. The average daily rate of 45 calcium increase of 52 cpm (66 cpm below the daily intake rate of the Controls and 11 cpm below that of the Low calcium samples), was the lowest recorded for any of the experimental populations, and was associated with the greatest increase in longevity value - further evidence for the inverse relationship between rate of calcium intake and longevity value. The greatest day increases in 45 calcium accumulated were again noted at the end of the life period, the final count representing the largest single day's 45 calcium accumulation during life, c.f. the Controls, while the total 45 calcium accumulated as a fraction of the total 45 calcium present in the culture medium was 0.11% lower than that of the untreated Controls, emphasising the lower count of 782 cpm in the group (278 cpm below the Controls), despite the relatively much longer exposure period. The rotifers that died in this group during citrate treatment


45 CALCIUM WITHDRAWN AS A %AGE OF TOTAL PRESENT BEFORE TREATMENT
0
10
20
30
40
50
60
70
80
90
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS OF LIFE

Graph 14

had in all except 2 cases accumulated at least 480 cpm, which was a comparatively high value when one considers that the corresponding washing counts representing the 45 calcium withdrawn from these rotifers ranged from 200 to 509 cpm.

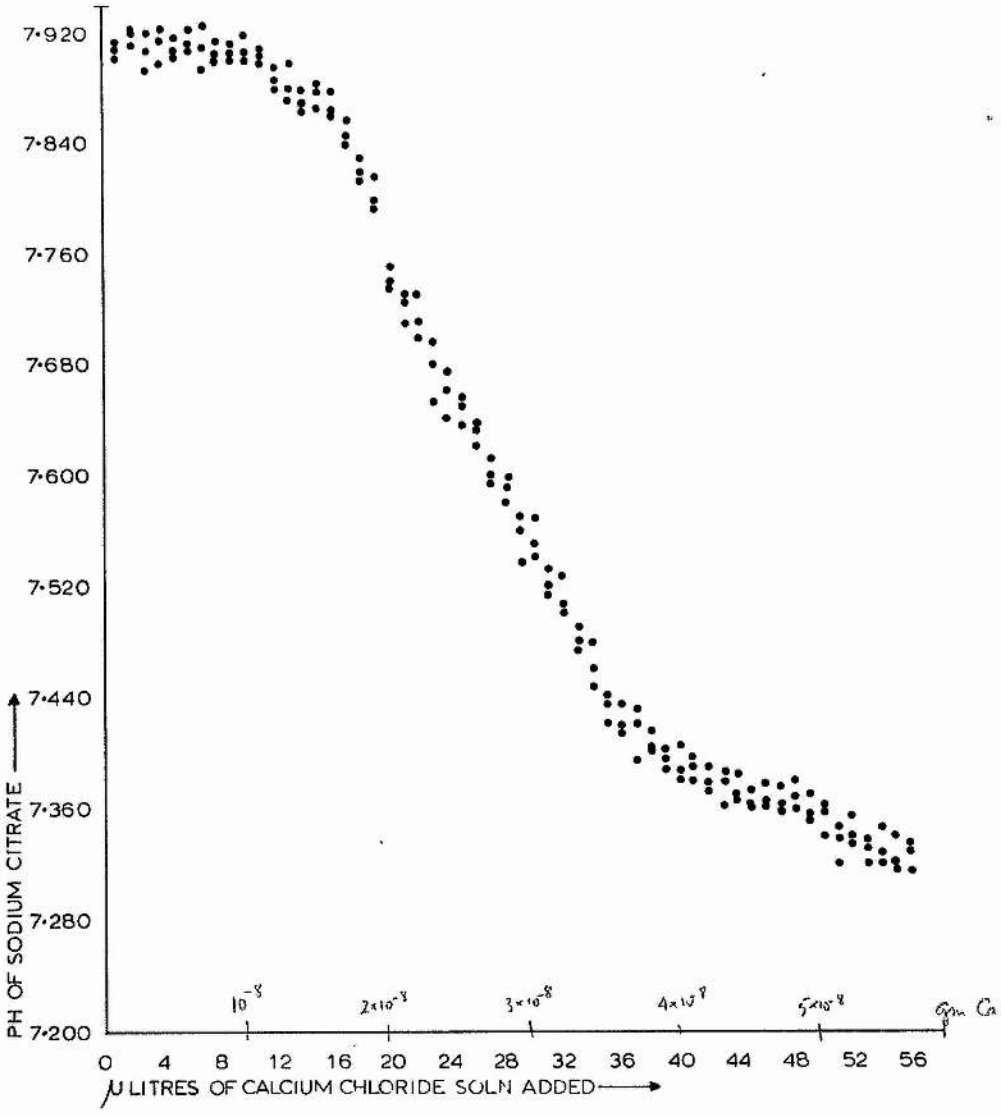
The total 45 calcium counts in the sodium citrate medium in which rotifers cultured on Control 45 calcium medium were briefly immersed on alternate days of life, showed a significant withdrawal of 45 calcium that began at day 3, the first day of recorded counts for the untreated Controls and continued on each day of treatment throughout the life period. The 45 calcium withdrawn on each washing day as a percentage of the total 45 calcium present in rotifers before immersion in sodium citrate, decreased at each successive treatment throughout life (see Graph 14), with a particularly marked decrease of 39% occurring at day 5, and a less marked reduction of about 15% occurring at day 9, the percentage reductions on all remaining days being less than 5%. The percentage fraction of the total 45 calcium removed by a chelating agent may be taken as an index of the combined efficiency of the chelating agent as a specific remover of calcium and the ease with which the calcium itself may be displaced from the particular site at which it has accumulated in rotifer tissues. Given that the efficiency of the chelating agent as a remover of calcium in the same state of binding and accessibility did not change under the standard conditions of the experiment (providing the number of ligands always exceeded the number of calcium ions), it is most likely that the strength of binding and accessibility of the accumulated calcium constituted the major source of variation in the amounts of calcium withdrawn throughout life. At day 5, the 39% reduction in the withdrawal of calcium could imply that the given efficiency of the chelating agent was not sufficient to overcome an increase in the amount of permanently bound calcium located possibly at a more inaccessible site of accumulation. The smaller 15% reduction in calcium withdrawal at day 9, could in turn indicate a relatively smaller reduction in the amount of calcium that could be exchanged at chelation. On the basis of this same argument it is tempting to

conclude that the relatively small reduction in the percentages of calcium removed on the other days of life (which included the period of later life), indicated relatively high proportions of exchangeable calcium in the total calcium accumulated on these days. It is also interesting to note that the 39% reduction in exchangeable calcium at day 5, occurred at the first accumulation of 45 calcium in the citrate treated group and in the early stages of a consistently increasing accumulation in the untreated Controls, while the smaller reduction at day 9, occurred on the same day that a marked 45 calcium increase was evident in the citrate treated Controls and the largest increase in calcium accumulated during the entire sampling period was recorded for the untreated Control group. In making comparisons between the percentages of calcium withdrawn and the calcium accumulated in the untreated Controls, it must be recalled that citrate treated rotifers showed a different calcium accumulation pattern in which a marked reduction of calcium accumulation compared to the untreated group occurred even when the amounts of calcium withdrawn during citrate treatment were added to the amounts of calcium accumulated between each washing day.

MATERIALS AND METHODS

pH CHELATION EXPERIMENT

As a means of providing additional information regarding the total amount of calcium withdrawn from rotifers by treatment with the chelating agent sodium citrate, an experiment was designed in which the pH changes undergone by the citrate washing volume at each chelation treatment were measured by a combined micro-electrode, Russel Electrode Ltd, and a 5 digit pH meter (Radiometer, Copenhagen). The bulb of the combined calomel and reference micro-electrode was specially constructed to measure the pH of 0.04 ml volumes in a well of plastic (glass would have been unsuitable as calcium ions from its calcium based silicates could be withdrawn by the chelating agent). Rotifers were cultured in 45 calcium Control medium in exactly the


7·920
7·840
7·760
7·680
7·600
7·520
7·440
7·360
7·280
7·200
PH OF SODIUM CITRATE
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56
μ LITRES OF CALCIUM CHLORIDE SOLN ADDED
gm Ca

Graph 15

same manner as the citrate treated population in the Ion Ratio Experiment. On alternate days rotifers were washed individually in 2 separate volumes of double distilled de-ionised water before being transferred individually to separate wells containing 0.04 ml volumes of a 0.50% solution of unbuffered sodium citrate. After 45 seconds of immersion rotifers were removed from the wells without withdrawing washing fluid and washed individually in 2 separate 0.04 ml volumes of Control 40 calcium culture medium, before being transferred back to fresh individual Control cultures. The combined micro-electrode was then lowered into the individual plastic wells and the changes undergone by the sodium citrate washing volumes recorded, the combined micro-electrode being carefully rinsed in double distilled de-ionised water between individual measurements. Before changes in pH undergone by the unbuffered volumes of sodium citrate could be converted into weights of the total ions removed from rotifers, it was necessary to plot a calibration curve. To a 10 ml volume of unbuffered 0.5% solution of sodium citrate was added, in one microlitre aliquots, a standard calcium solution containing one microgram of calcium per millilitre as chloride. At each addition of the standard solution the sodium citrate was stirred thoroughly before a 0.04 ml volume was withdrawn to a plastic well where its pH could be measured under identical conditions to those in the experiment. This procedure was repeated until the calibration curve covered the range of pH changes undergone by the experimental washing volumes. The weights of ions withdrawn, that corresponded to the weights of a standard calcium solution added to sodium citrate could then be read off the appropriate parts of the calibration curve, (see Graph 15).

RESULTS

pH CHELATION EXPERIMENT

The pH changes recorded in sodium citrate indicated a significant exchange between the protons of the chelating agent and the total divalent ion species withdrawn from rotifers on each day of treatment (see Table 11). On day 3, the first day that a population of 20

TABLE 11

pH CHELATION EXPERIMENT	
DAY OF TREATMENT	CORRESPONDING WEIGHTS OF CALCIUM REMOVED
3	22.8×10^{-12} gms
5	22.8×10^{-12} gms
7	25.2×10^{-12} gms
9	26.0×10^{-12} gms
11	32.1×10^{-12} gms
13	34.3×10^{-12} gms
15	52.1×10^{-12} gms

rotifers were briefly and individually immersed in separate unbuffered volumes of chelating agent, an average pH change was produced that corresponded to a total weight of 22.80×10^{-12} gms of calcium. Thereafter, the pH changes undergone by fresh sodium citrate volumes at each successive day of rotifer immersion up till day 15, corresponded to a slow increase in the equivalent weights of calcium that never exceeded 3×10^{-12} gms between any two washing days, with the exception of day 11 when an equivalent weight increase of 6.10×10^{-12} gms was recorded. At day 15, the final day of rotifer immersion, the average pH change undergone by the unbuffered chelating agent corresponded to 52.10×10^{-12} gms of calcium, representing a 17.8×10^{-12} gms increase from the previous washing day value, the largest increase recorded for any single day of treatment. The pH changes undergone by unbuffered sodium citrate on each day of washing from days 3 to 15 inclusive, corresponded to a range of from 22.80×10^{-12} gms to 52.10×10^{-12} gms in the equivalent weights of calcium.

PRELIMINARY DISCUSSION

pH CHELATION EXPERIMENT

The pH changes undergone by unbuffered sodium citrate in this experiment reflect the total quantity of divalent ion species withdrawn from rotifers by the ligands of the chelating agent. The pH change reflecting both these aspects of chelation has been compared to an equivalent weight of calcium on the grounds that the highest first order log b value of the formation constants between sodium citrate and divalent ions occurs between sodium citrate and calcium (see Table 7). Nevertheless, it is important to remember that all weights of calcium relating to pH changes include a small fraction of other divalent ion species (principally Mg^{++}).

The principle of the pH chelation experiment is that with the increasing occupation of the O^- Ligands of sodium citrate by divalent ions a corresponding increase will occur in the number of protons liberated from the chelating agent back into solution. The increases in the amounts of free protons will cause the pH of the citrate washing volume to become increasingly acidic, and it is these changes towards acidity that are used to estimate the weight of divalent ions withdrawn from rotifers by the ligands of the chelating agent.

It has already been mentioned that the pH changes undergone by sodium citrate reflect increases in the total quantity of divalent ions withdrawn. By way of contrast, the previous radioactive citrate experiment reflected only the increases of a small labelled fraction of withdrawn calcium. In this context, it is interesting to note that the equivalent weights of calcium withdrawn on each day of treatment in the present experiment exceeded the corresponding day weights of 45 calcium in the Radiotracer Experiment 1, since calcium weights reflecting pH changes included a small fraction of other divalent ion species.

The overall pattern of calcium withdrawal in the pH experiment reflected a gradual increase throughout most of the life period, with a marked increase in the weight of ions withdrawn at day 11, and a further particularly high increase at day 15, the final day of treatment. By way of comparison the pattern of 45 calcium withdrawal again reflected a gradual increase up till day 11, when a marked increase in the 45 calcium withdrawn was also noted. This increase continued till the end of life with a higher increase in the weight of 45 calcium removed being recorded on day 13, but again a significantly large weight of calcium being withdrawn on the final day of treatment.

TABLE 12

CULTURE TRANSFER EXPERIMENT

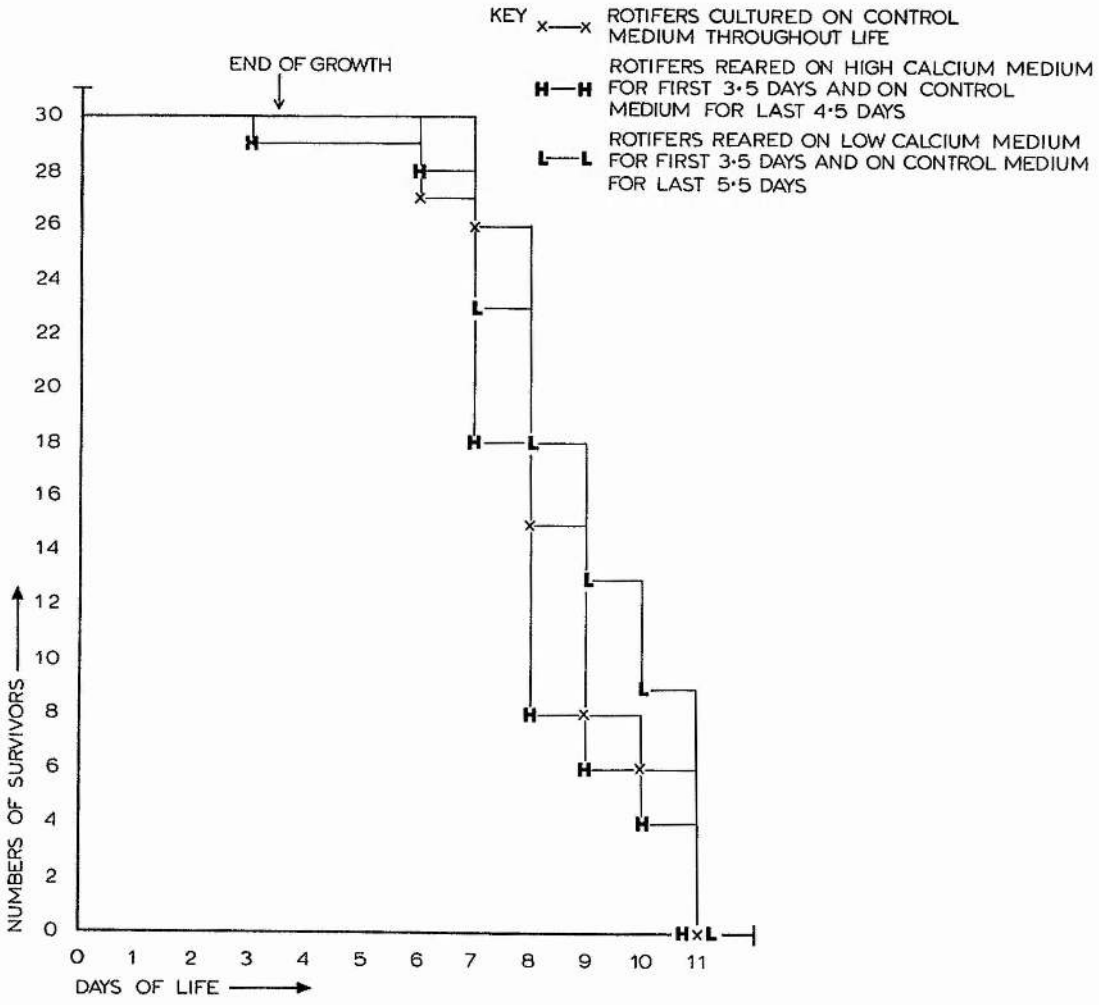
GRP sub grpsset	1st 3.5 days	Remainder of Life	mean life span
90 (30)(30)(30) --- 1	H	C	8.0 days
	L	C	9.0 days
	C	C.... CONTROLS	8.7 days *
90 (30)(30)(30) --- 2	C	H	8.0 days
	L	H	8.5 days
	H	H.... CONTROLS	7.7 days *
90 (30)(30)(30) --- 3	H	L	12.0 days
	C	L	12.7 days
	L	L ....CONTROLS	12.6 days *

* denotes later life Control

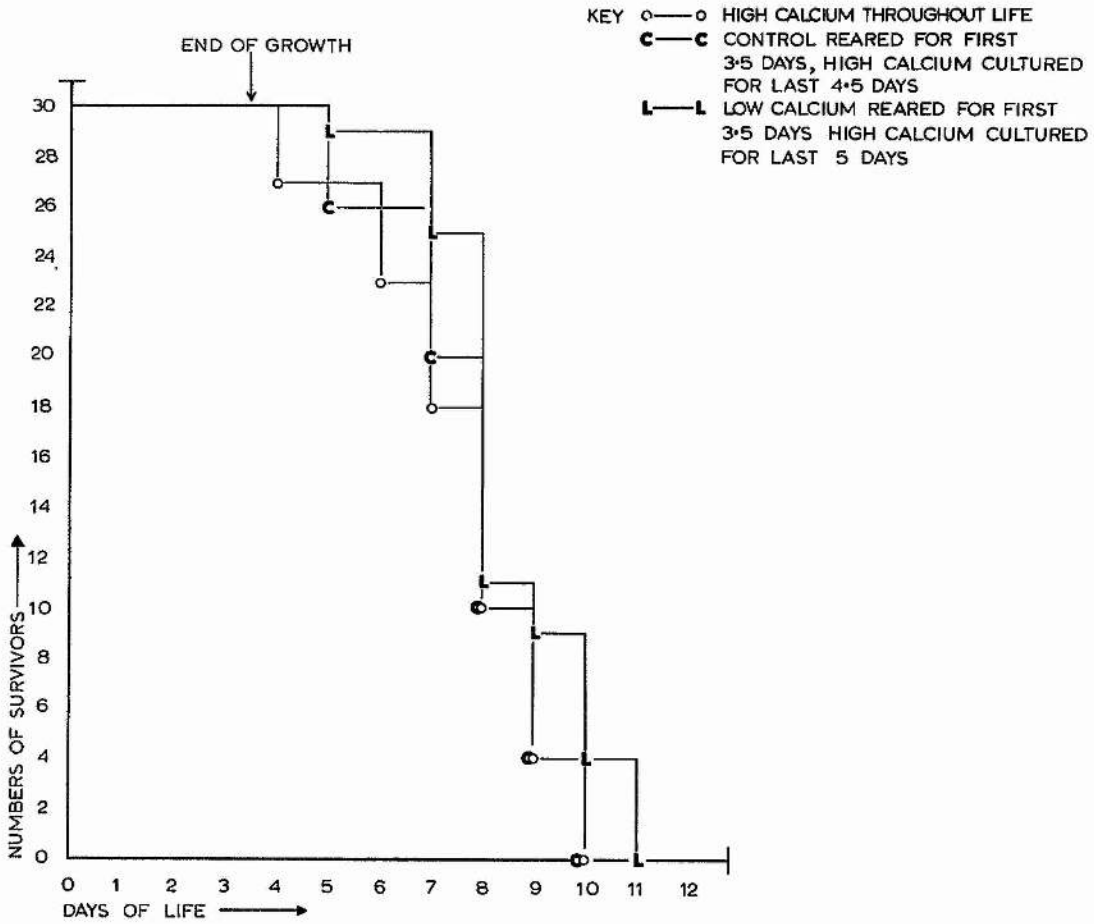
TABLE 13

CULTURE TRANSFER EXPERIMENT

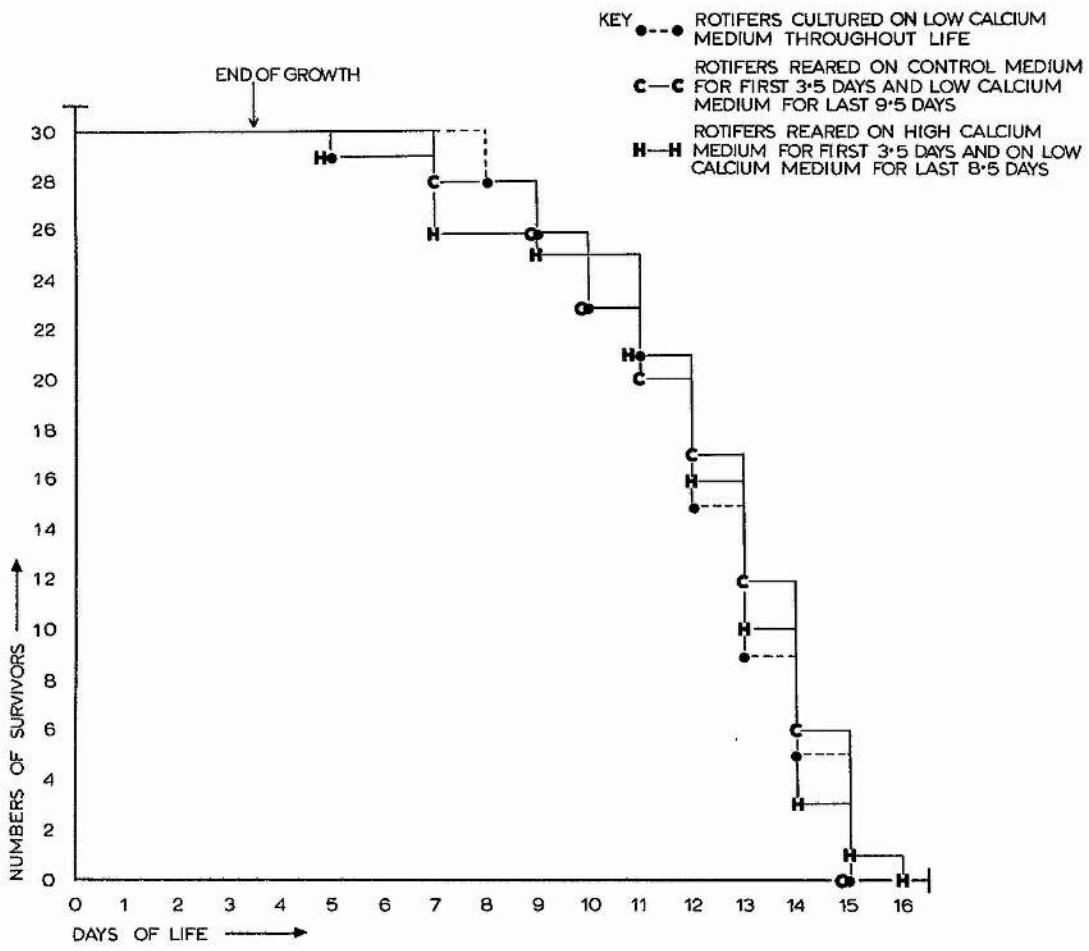
POPULATION	MEAN LONGEVITY	DIFFERENCE	TOTAL EGGS
CONTROLS	8.7 days S.E. $\pm$ 0.2 days		133
LOW TO CONTROL	9.1 days S.E. $\pm$ 0.2 days	4.6%	146
HIGH TO CONTROL	8.0 days S.E. $\pm$ 0.3 days	8.0%	106
HIGHS	7.6 days S.E. $\pm$ 0.2 days		99
LOW TO HIGH	8.6 days S.E. $\pm$ 0.2 days	11.6%	100
CONTROL TO HIGH	7.9 days S.E. $\pm$ 0.2 days	3.9%	103
LOWS	12.2 days S.E. $\pm$ 0.3 days		210
CONTROL TO LOW	12.3 days S.E. $\pm$ 0.3 days	0.82%	239
HIGH TO LOW	12.0 days S.E. $\pm$ 0.3 days	1.6%	209


KEY
ROTIFERS CULTURED ON CONTROL MEDIUM THROUGHOUT LIFE
ROTIFERS REARED ON HIGH CALCIUM MEDIUM FOR FIRST 3·5 DAYS AND ON CONTROL MEDIUM FOR LAST 4·5 DAYS
ROTIFERS REARED ON LOW CALCIUM MEDIUM FOR FIRST 3·5 DAYS AND ON CONTROL MEDIUM FOR LAST 5·5 DAYS
END OF GROWTH
NUMBERS OF SURVIVORS
DAYS OF LIFE
0 1 2 3 4 5 6 7 8 9 10 11
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
H×L

Graph 16


KEY o—o HIGH CALCIUM THROUGHOUT LIFE
C—C CONTROL REARED FOR FIRST 3·5 DAYS, HIGH CALCIUM CULTURED FOR LAST 4·5 DAYS
L—L LOW CALCIUM REARED FOR FIRST 3·5 DAYS HIGH CALCIUM CULTURED FOR LAST 5 DAYS
END OF GROWTH
NUMBERS OF SURVIVORS
DAYS OF LIFE
0 1 2 3 4 5 6 7 8 9 10 11 12
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Graph 17


KEY
ROTIFERS CULTURED ON LOW CALCIUM MEDIUM THROUGHOUT LIFE
ROTIFERS REARED ON CONTROL MEDIUM FOR FIRST 3·5 DAYS AND LOW CALCIUM MEDIUM FOR LAST 9·5 DAYS
ROTIFERS REARED ON HIGH CALCIUM MEDIUM FOR FIRST 3·5 DAYS AND ON LOW CALCIUM MEDIUM FOR LAST 8·5 DAYS
END OF GROWTH
NUMBERS OF SURVIVORS
DAYS OF LIFE
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Graph 18

MATERIALS AND METHODS

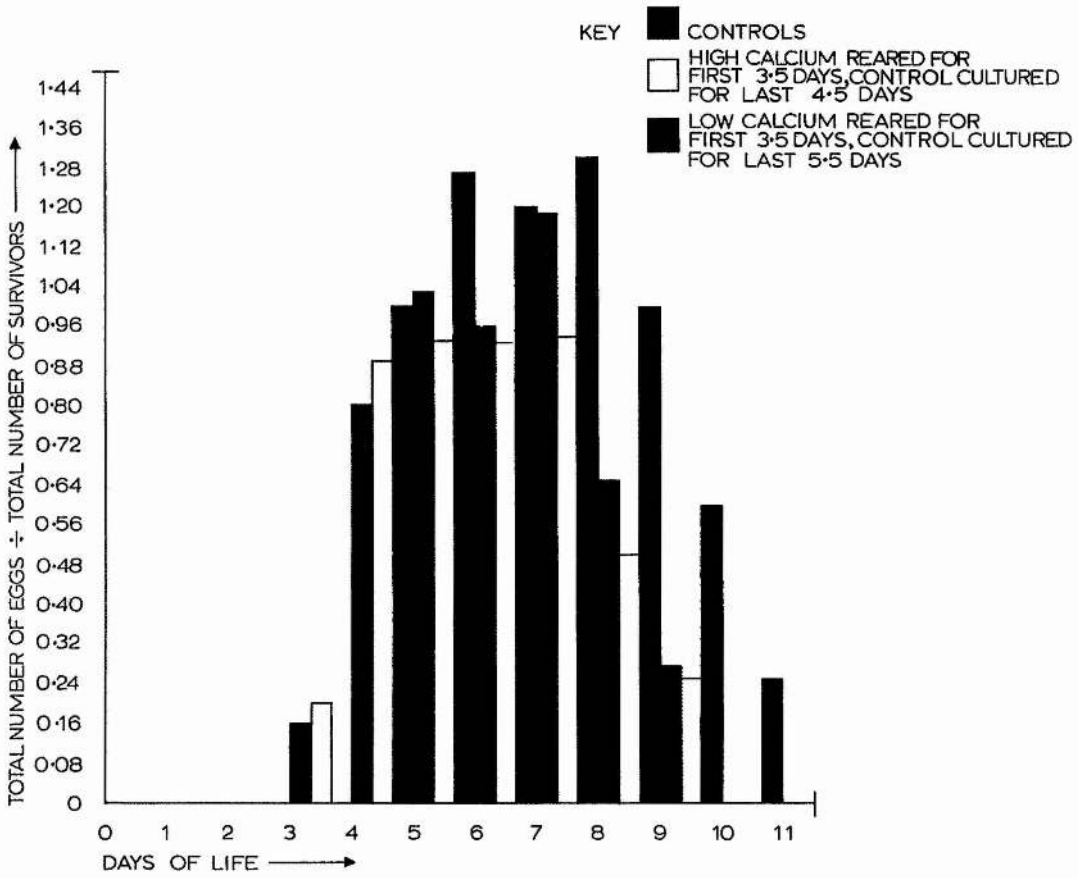
CULTURE TRANSFER EXPERIMENT

A final group of ion ratio experiments was designed to supplement those of the Ion Ratio Experiment. Three groups of 90 individually cultured rotifers were established each with three sub groups of 30 being grown for the first 3.5 days of life on one of the three modified Knop's media employed in the Ion Ratio Experiment. Half way through the third day when all growth had taken place, two sub groups out of each set of three were transferred to a different medium of the three for the remainder of their life period. This was done in such a way that two out of the first set of three sub groups ended their life in Control medium, two out of the second set in High calcium medium, and two out of the third set in Low calcium medium. The remaining third sub group of the three was employed as a later life Control and consisted of rotifers cultured throughout life in the medium to which the other two sub groups in the set were transferred at day 3. The procedure is summarised in Table 12. Before each transfer at day 3 all rotifers were washed thoroughly in the medium to which they were being transferred.

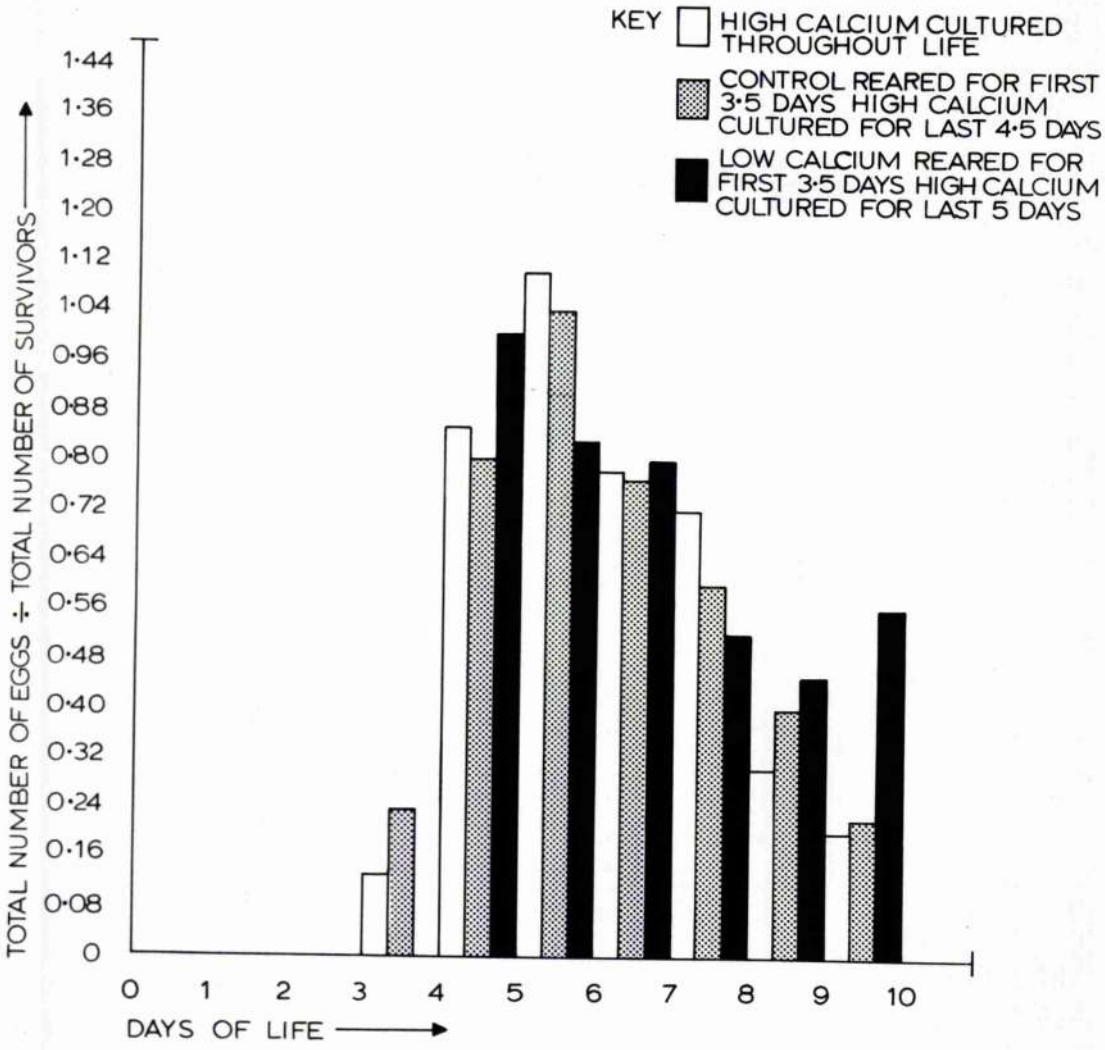
RESULTS

CULTURE TRANSFER EXPERIMENT

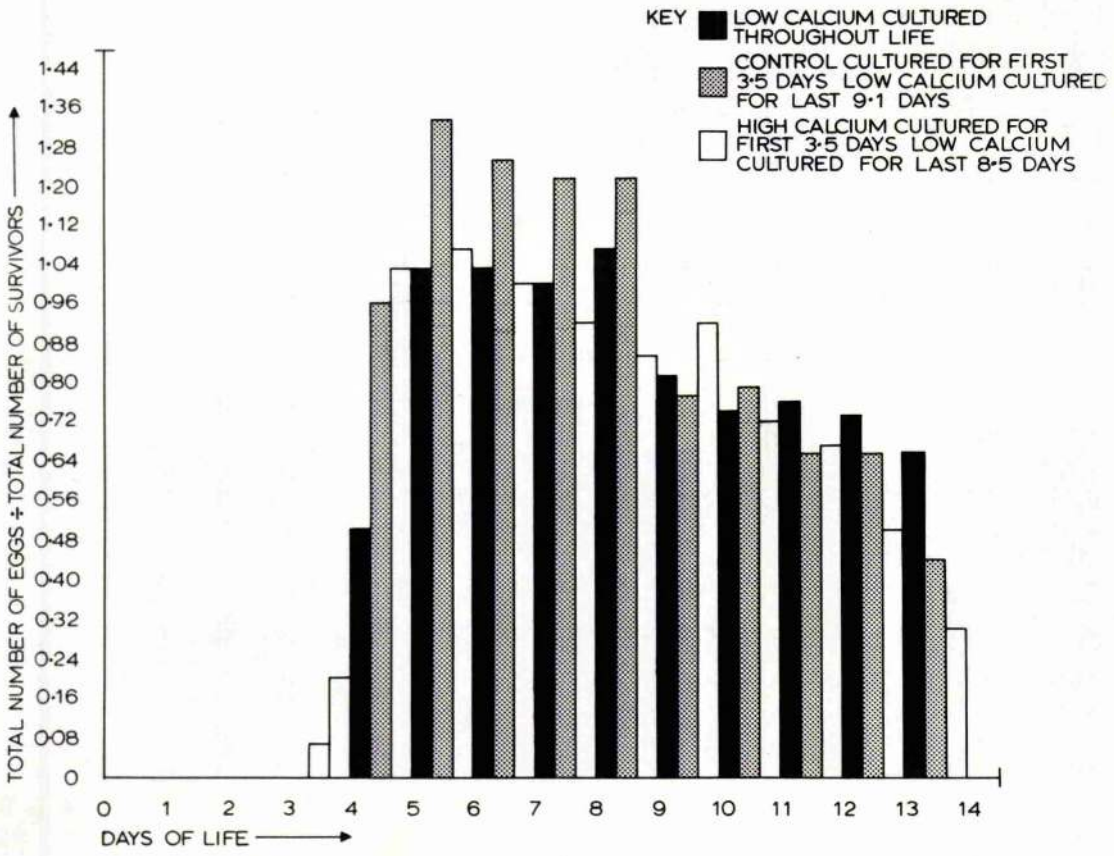
The two sub groups of 30 rotifers in Set 1 that were transferred on day 3 of life from High and Low calcium media to spend the remainder of their life in Control medium, show an overall mean longevity value that approximated to that obtained for rotifers in Set 1 that spent their entire life period in Control calcium medium (see Graph 16 and Table 13). Likewise, rotifers in Sets 2 and 3 that were transferred to High and Low calcium media respectively at day 3, show overall life-spans that approximate to those shown by rotifers that spent their entire life period in High and Low calcium media (see Graphs 17 and 18 and Table 13). This would indicate that it was not the media on


KEY
CONTROLS
HIGH CALCIUM REARED FOR FIRST 3·5 DAYS, CONTROL CULTURED FOR LAST 4·5 DAYS
LOW CALCIUM REARED FOR FIRST 3·5 DAYS, CONTROL CULTURED FOR LAST 5·5 DAYS
TOTAL NUMBER OF EGGS ÷ TOTAL NUMBER OF SURVIVORS
1·44
1·36
1·28
1·20
1·12
1·04
0·96
0·88
0·80
0·72
0·64
0·56
0·48
0·40
0·32
0·24
0·16
0·08
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS OF LIFE

Histogram 4


KEY
HIGH CALCIUM CULTURED THROUGHOUT LIFE
CONTROL REARED FOR FIRST 3·5 DAYS HIGH CALCIUM CULTURED FOR LAST 4·5 DAYS
LOW CALCIUM REARED FOR FIRST 3·5 DAYS HIGH CALCIUM CULTURED FOR LAST 5 DAYS
TOTAL NUMBER OF EGGS ÷ TOTAL NUMBER OF SURVIVORS
1·44
1·36
1·28
1·20
1·12
1·04
0·96
0·88
0·80
0·72
0·64
0·56
0·48
0·40
0·32
0·24
0·16
0·08
0
0 1 2 3 4 5 6 7 8 9 10
DAYS OF LIFE

Histogram 5


KEY
LOW CALCIUM CULTURED THROUGHOUT LIFE
CONTROL CULTURED FOR FIRST 3·5 DAYS LOW CALCIUM CULTURED FOR LAST 9·1 DAYS
HIGH CALCIUM CULTURED FOR FIRST 3·5 DAYS LOW CALCIUM CULTURED FOR LAST 8·5 DAYS
TOTAL NUMBER OF EGGS ÷ TOTAL NUMBER OF SURVIVORS
1·44
1·36
1·28
1·20
1·12
1·04
0·96
0·88
0·80
0·72
0·64
0·56
0·48
0·40
0·32
0·24
0·16
0·08
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS OF LIFE

Histogram 6

which rotifers were cultured during the growth period that exerted the major effect in determining life-span, but the media to which rotifers were transferred after the growth phase had been completed.

The duration of egg-laying corresponded to the duration shown by the later life Controls (see Histograms 4, 5 and 6), although in one case, that of the population that began life in Low calcium medium and ended it in Control medium, egg-laying continued for one day longer than was the case in the Control group (see Histogram 4). The 24 hour delay in onset of egg-laying previously noted in Results, Ion Ratio Experiment Page 44 , was again evident in populations that began their life in Low calcium media. The total number of eggs laid by each sub group corresponded to the values obtained for the later life Controls in their respective Set (see Table 13), although it is interesting to note that sub groups in Sets 1 and 2 that began their life in Low calcium media showed slightly improved egg-laying in later life. In no set was a reduction of egg-laying level recorded that was consistently below that of the Control sub group in Set 1.

PRELIMINARY DISCUSSION

CULTURE TRANSFER EXPERIMENT

It is noteworthy that rotifer sub groups that underwent their growth periods in High calcium medium showed mean longevity values that although they approximated to those of their later life Controls, showed more variability than rotifers that spent their growth periods in Control, and with one exception in Set 2, Low calcium media. This could be due to the fact that High calcium populations begin accumulating calcium on days 3 and 4 at a significantly higher rate than in other groups, (see Table 8) consequently a small but significantly higher calcium intake may have occurred at the end of the growth period before transfer to later life media, producing a slight reduction in life expectancy in these sub groups. It is also interesting to note that the one exceptional case in Set 2, the sub group that underwent growth in Low calcium medium and then spent the remainder of its life in High calcium medium, showed a slight extension of its life period compared to the later life Controls,

which may have resulted from the significantly low accumulation rate of calcium in the Low calcium medium at the end of the period of growth (see Table 8).

DISCUSSION - PART 1

Lansing in his 1942a paper investigated the effects of calcium on the longevity and egg-laying of Rotifer vulgaris, by culturing individuals on the same three modified Knop's media as were employed in the Ion Ratio Experiments. The results he obtained were similar to those in this present study, with a 33% increase in life expectancy in the population cultured on Low calcium - High potassium medium, but no reduction in life expectancy in the High calcium - Low potassium group. In this present work a 40% increase in life expectancy was found for the rotifer Mytilina brevispina var redunca cultured on Low calcium - High potassium medium, and in addition a 13% reduction in life expectancy was obtained for the High calcium - Low potassium group. Lansing also achieved a considerable increase in life expectancy of 43% in a Control cultured group of the species Proales sp. by subjecting individuals to brief immersions in a 0.5% solution of sodium citrate on days 2, 4, 6, 7, 8 and 9 of life. An increase in life expectancy of 52% was obtained in this present study for individuals of the species Mytilina brevispina var redunca cultured on the same Control medium and subjected to brief immersions in 0.5% sodium citrate solution on alternate days of life beginning at day 3. Lansing concluded at the end of his 1942a paper that in the absence of genetic variation, the rotifers used in both of his studies and my own reproduced entirely by ameiotic diploid parthenogenesis, the accumulation of calcium could be a significant factor in rotiferan ageing, since rotifers cultured in Low calcium - High potassium Knop's medium and rotifers subjected to individual brief immersions in sodium citrate (which removed calcium), showed significant increases in life expectancy. It should be emphasised that this conclusion relied heavily on the assumption that sodium citrate removed only

calcium from rotifers, no comparisons being made between the log b values of the formation constants between sodium citrate and calcium and sodium citrate and other divalent ion species. It was for this reason that further work was carried out in this present study which employed such chelating agents as E.G.T.A. and E.D.T.A., which exhibit a high specificity for calcium in comparison with magnesium, the other divalent ion species most likely to be withdrawn by the chelating agents used by Lansing and myself. It was found in this present study that treatment on alternate days of life with a 0.25% solution of E.G.T.A., which has a 10^6 X greater specificity for calcium than magnesium, produced an increase in life expectancy of 76%, while a similar method of treatment employing E.D.T.A., which is 10^2 X more specific for calcium than magnesium, produced an increase in life expectancy of 49%. This direct relationship between the specificity of a chelating agent for calcium (estimated in terms of log b value of the resultant calcium - chelating agent complex), and resultant increase in life expectancy, was further borne out in experiments employing other chelating agents i.e. sodium tartrate. Sodium tartrate has a very low specificity for calcium (see Table 7), and the resultant life expectancy which it produced (corresponding to a 44% increase) was significantly below that of the 76% increase produced by E.G.T.A., though not so significantly below that of the 49% increase associated with E.D.T.A., which has a relatively higher calcium specificity. This disparity emphasises the fact that many chelating agents most specific for calcium also exhibit increased toxicity towards the rotifer species treated. In the case of Mytilina brevispina var redunca E.D.T.A. was found to be particularly toxic, such that a doubled time of immersion or a doubled concentration of this chelating agent produced instant death, whereas by way of contrast rotifers could tolerate either of these increases in the case of the other chelating agents employed in this study.

The calcium specificity of sodium citrate at approximately double the ionic strength of the other chelating agents, is 2.5 X greater than its specificity for magnesium, (see Table 7). It was

associated with a 52% increase in life-span, a significantly greater increase than was produced by sodium tartrate (which has a lower calcium specificity at approximately half the ionic strength), and about the same increase in life expectancy as was produced by E.D.T.A. (which has a far greater calcium specificity at approximately half the ionic strength). In terms of the direct relationship between increase in life expectancy and the calcium specificity of the chelating agent, E.D.T.A. would appear to produce an unexpectedly low life increase, however as was mentioned previously this could be accounted for on the grounds that E.D.T.A. exhibited both a beneficial and toxic effect.

In order to test the hypothesis that increased calcium is a significant factor in rotiferan ageing the series of radiotracer studies were undertaken to investigate the patterns of postulated calcium uptake in each of the three rotifer populations reared on the three modified Knop's media, and the pattern of postulated calcium withdrawal in rotifers subjected to treatment with the chelating agent sodium citrate. With these aims in view care was taken to design a standardised radiotracer procedure that did not induce radiation damage and did not permit the external contamination of samples with the radionuclide under investigation. The absence of radiation damage and variability in a Control 45 calcium cultured population of 30, was shown by its similar growth rate, egg-laying, and mean longevity value to a Control population of the same number cultured on 40 calcium medium. Similarly, no variability in the characteristics of the Control 45 calcium cultured progeny was noted over three generations of 45 calcium culture. Care was also taken in all radioactive procedures to ensure that all experimental samples were counted as many times as were required to give an average count above background level that was statistically significant at all levels of counting. All samples in a given radioactive procedure were in fact counted 10 times on the same day at the end of the experiment. This counting number ensured that all counts higher than 50 cpm above background were significant at about the 1% level. The level of significance was not as high for counts below 50 cpm,

and such sample counts in themselves could not be taken as so critical a measurement of 45 calcium. However only one count of this particularly low magnitude was obtained, and the prime purpose of all radioactive procedures anyway was to provide comparative information regarding the general pattern of calcium movement in differently cultured populations.

The results of the rotifer populations cultured in Control, Low and High calcium radioactive media showed an accumulation of 45 calcium in the manner predicted by the Lansing hypothesis. No accumulation was recorded in any population until the end of the period of growth, and the accumulation that was subsequently recorded in each of the three populations occurred at a far slower rate in the population cultured on Low calcium medium which showed an increased life expectancy. High calcium cultured rotifers showed an initial accumulation of calcium that was higher than that recorded for the Controls, and although the calcium accumulation rate thereafter was approximately the same as that of the Control group for the majority of the life period, this initially higher accumulation produced a consistently higher count on any day of life. This, in conjunction with a final high increase in rate of 45 calcium accumulation that occurred one day before a similar increase in the Controls, could have explained the 13% reduction in mean longevity recorded for the High calcium cultured group. Most of the rotifers that died on the same day that they were sampled in all of the three populations showed a high calcium content at death.

The critical test of the calcium accumulation theory is to be found in the radioactive chelation experiments. These consisted of counting 45 calcium in the sodium citrate washings of treated rotifers, and in a second experiment counting the citrate treated rotifers themselves. The washings of the citrate treated animals showed significant quantities of withdrawn 45 calcium that increased steadily throughout life, though most notably after day 11, while the results of the citrate treated rotifers indicated a much slower 45 calcium accumulation than the untreated Controls, with a slow steady increase throughout life though most notably after day 13, when a final increase corresponding to 274 cpm per day was noted.

These results confirm Lansing's statement in his 1942a paper that significant quantities of calcium were removed from rotifers during the time that they were immersed in a solution of sodium citrate. A pH method of estimating the total weight of divalent ions withdrawn at treatment with sodium citrate also revealed significant increases throughout the life period. Compared to the 45 calcium citrate treatment results, which only reflected the withdrawal of a specific and small labelled fraction of the total divalent ions accumulated, the pH method reflected the expectedly larger estimate of the total complement of divalent ions exchanged.

The calcium withdrawn as a percentage of the total calcium present in rotifers before citrate treatment decreased throughout life, with a particularly marked decline at day 5, and a smaller reduction at day 9. It was postulated earlier that these reductions could reflect increases in the fraction of calcium that was permanently bound within rotifer tissues, and hence could not readily be exchanged at chelation. The significance of the day 5 reduction is considered again later in this discussion.

The relation of the calcium ageing hypothesis to growth in the rotifer has been described in the INTRODUCTION, with respect to the particular rotifer species studied. The findings that calcium did not appear in micro-incinerated sections of the rotifer Euchlanis dilatata until growth had been completed (Lansing 1942b), and also the discovery that the change from pediaclone to geriaclone in the rotifer Philodina citrina occurred at the end of the growth period (Lansing 1948), led Lansing to associate the appearance of his ageing factor (calcium) with the time of cessation of growth. It is interesting to note that this hypothesis is supported by the results of the radiotracer experiments undertaken in this study, which indicate that no significant intake of 45 calcium occurred until the end of the growth period, growth being here defined as increase in body size. It has been pointed out by Meadow and Barrows (1969), that in the rotifer Philodina acuticornis marked increases in dry weight and protein content occurred during the post-reproductive

period, when a strong force of mortality was in evidence, and this would not support Lansing's view that ageing in rotifers is the result of cessation of growth, if growth is defined as an increase in dry mass. However, it is quite clear in Lansing's 1948 paper that growth cessation referred to cessation of increase in body size, and not body dry mass.

The results of the culture transfer experiments in which rotifers were grown on one of the three modified Knop's media during the growth period and transferred to another of the three media for the remainder of life, demonstrated that it was not the medium on which rotifers were grown that exerted the major effect in determining longevity, but instead the medium to which rotifers were transferred immediately after the growth phase had been completed. This overall result is in keeping with the Lansing hypothesis that the ageing factor was not operative until the cessation of growth, and is also in agreement with the results obtained in the radioactive procedures where rotifers cultured during growth on the three modified 45 calcium media showed no significant accumulation of 45 calcium until the end of the growth phase. However, slight variations that were in agreement with the magnitude of 45 calcium intake at the end of growth were found in the culture transfer experiments, (see Preliminary Discussion Culture Transfer Experiment Page 93), though these were sufficiently slight to have little effect on the broad conclusions drawn from experimental results.

It is particularly interesting to note that after the end of the period of growth in size, the 45 calcium withdrawn from rotifers by sodium citrate as a percentage of the total 45 calcium present in rotifers before washing, was 100%, indicating the presence of no permanently bound 45 calcium at this time. However, on the following day of treatment day 5, a 39% reduction in the percentage of withdrawn 45 calcium was noted, and this may have reflected an increase in the fraction of inexchangeable calcium that began after the final increase in body size was recorded. The question arises as to whether

this increase in the postulated permanently bound calcium fraction was the initially accumulated calcium that Lansing proposed marked the point of change from pediaclone to geriaclone in his orthoclone studies. Certainly an increase in permanently bound calcium at this time of life (between days 3 and 5) could exert a particularly strong effect on longevity, particularly if it was accumulated in the manner Lansing proposed in 1948 between generations. Further 45 calcium orthoclone studies would have to be undertaken to investigate this point, however it is significant that in the matrocliny experiment described on page 29 for the species Mytilina brevispina var redunca, a slight reduction in life-span and egg-total was noted after 2 generations of selection in the 5 day orthoclone, and this may have corresponded to the point in life where an increase in the fraction of permanently bound calcium effected the change from pediaclone to geriaclone in this species.

It was pointed out by Lansing in 1954 that a characteristic of a pediaclone (an orthoclone that exhibits increased longevity over successive generations of selection) was a shift in the time of onset of egg production to later ages. This is of particular significance in relation to the populations in Part 1 of this study that exhibited increases in life expectancy, since all of them displayed a delayed onset of egg-laying, with an especially marked delay being recorded in the case of the population treated with E.G.T.A., which showed a 76% increase in longevity.

Lansing 1948 observed in the case of Philodina citrina that animals became increasingly granular in appearance with age, and it was even implied that this granularity was of a calciferous origin. Other authors Pray, Ferris and Chu have noted that representatives of both Bdelloid and Monogonont Classes became increasingly transparent with age. In the case of Mytilina brevispina var redunca no age related granular changes in appearance were noted, however some individuals did display an increased transparency in old age.

Although comparisons have been made between Lansing's results and those of this present study it should be noted that differences do exist between them concerning the degree of standardisation of experimental culture procedures. Lansing did not work under the strictly aseptic conditions that prevailed in this present work, which is particularly significant in the light of the results of Meadow and Barrows 1969 that confirm my own that variability in longevity value can readily result from uncontrolled bacterial components in individual cultures. Secondly, Lansing did not carry out a selection procedure to derive all experimental populations initially from a single parent and then homologise them with respect to maternal age over a fixed number of generations, thereby eliminating the sort of matroclinous variation that was the subject of his 1947, 1948 and 1954 papers. Likewise, in none of Lansing's studies were the algal components of individual cultures monitored and standardised, or temperature variations undergone by cultures maintained to within a range of $1^{\circ}C$. Since it was discovered before experimentation in this present study that overfeeding resulted in marked reductions in egg-laying, and underfeeding could produce alterations in longevity value, the algal component of individual cultures was controlled to within 50 cells between cultures by micro-nephelometer standardised against a haemocytometer. The temperature variations undergone by individual cultures in this work were maintained to within $\pm 0.2^{\circ}C$, particularly as Meadow and Barrows 1969 have shown that variations of the order of $1^{\circ}C$ can produce longevity variations in the rotifer Philodina acuticornis of up to 3 days. Finally, a selection procedure to eliminate matroclinous variations and the preservation of sterile culture conditions to eliminate varied bacterial components in cultures, were all aimed at standardising the procedures in these present experiments, thereby eliminating the sort of variations that could obscure true ageing phenomena.

While it has been emphasised that Lansing's theory of rotiferan ageing is in agreement with the results of this present study, it is important to recognise that this is not the only theory of rotiferan ageing to be advanced. Miller in 1931 proposed that death occurred in rotifers as a result of reproductive exhaustion. Her hypothesis was

based on the fertility and longevity of the rotifer Lecane inermis.[1] The mictic form of this species has a short reproductive period, egg-laying ceasing in early life, and a longer mean longevity than the amictic form in which the reproductive period is relatively more prolonged. It should be pointed out that these reproductive differences are not always apparent in Miller's data, and it is on these grounds that Miller's theory may be criticised, (Comfort 1954). In 1932 Ferris noted differences in egg production between amictic and mictic forms of the rotifer Hydatina senta. In the case of this rotifer the amictic females had the greatest mean longevity and this was accompanied by a relatively lower rate of egg-laying in early life than the mictic forms, an observation which is in support of an ageing theory based on reproductive exhaustion, (Comfort 1954). Quite apart from the fact that the rotifers adopted in this present study were all amictic females, there was little evidence of shortened life-span as a result of differences in reproductive period. It is interesting to note that most of the populations in this study that displayed increased life expectancy also showed a slight delay in the onset of their reproductive period. However, this was not accompanied by a sustained lower rate of egg production than the untreated Controls (except in the case of the cysteine hydrochloride treated group described later), and the rate of egg-laying during later life more than compensated for this delay.

It has been stated by Brauer in Die Süsswasserfauna Deutschlands, that Mytilina brevispina var redunca reproduces only by ameiotic diploid parthenogenesis, no males or mictic phases having ever been observed in this rotifer in natural or artificial habitats. The genetic consequences of this form of parthenogenesis over succeeding generations of clones would be the complete absence of segregation and reconjugation of alleles, with the only source of genetic variation stemming from mutation. These speculations are entirely dependent on the accuracy of Brauer's statement and it would be unwise to rule out entirely the possibility of mixis in natural or artificial habitats, since this process may only need to occur very infrequently to provide the necessary "gene mix" for a great number of genetically viable clonal generations. It is important to bear in mind these genetic implications

1 Lecane inermis = Distyla inermis

of ameiotic diploid parthenogenesis when considering the more generally applicable Genetic theories of ageing in relation to rotifers.

There exists a great number of genetic theories of ageing, most of them being based on the age-related accumulation of genetic damage. Many of these theories predict the observed linear decline in vitality summarised in the Gompertz equation (Gompertz 1825) almost entirely by mathematical assumptions that are often not substantiated by qualitative and quantitative observations, i.e. the Brody-Failla and Simm-Jones theories. A theory of ageing based on genetic damage incurred exclusively as a result of somatic mutation has been propounded by Failla. Along with many other theories of ageing based on mutation it is not supported by a great deal of experimental evidence. For example Drosophila sp. exposed to 4500 rontgens of ionising radiation lived longer than the unexposed Controls (Sacher 1956), while Campanularia hydranths after exposure to 100,000 rontgens showed a 100% increase in longevity (Strehler unpublished). The unlikelihood of a theory of ageing based solely on somatic mutation could indicate that the main source of genetic variability in rotifers, namely mutation, is not in itself likely to exert a major influence on the ageing process, particularly as there exists a fixed cell number in these organisms.

Orgel in his 1963 paper proposed that the accumulation of protein transcription errors could constitute a source of progressive deterioration of cells and cell lines. Investigation of errors of this kind in rotifers by adding error inducing labelled amino acid analogues in sub toxic concentrations to the culture medium, i.e. p flourophenylalanine, and then relating the level of incorporation of the labelled analogues to longevity, were not carried out in this study. Certainly, experiments of this kind would be well worth performing on rotifers, since errors at the transcription level could exert a considerable effect on a clonally propagated species.

Sonneborn's studies on clonal ageing in Paramecium sp. indicate that autogamy (the equivalent of sexual crossing) is required in some species to maintain the vigour of succeeding generations of clones, while in other species clones may be successfully propagated without autogamy taking place. This may parallel the situation in rotifers where in some cases mixis regularly occurs during clonal propagation, while in others it has never been recorded. Sonneborn has carried out some interesting experiments on isolated clones of the flatworm Stenostomum incaudatum. When this organism undergoes fission it breaks into four pieces, each piece giving rise to a daughter individual. Sonneborn maintained separate lines of the fission products of this organism and discovered that clones which retained the original head parts senesced and died out, while clones derived from the original posterior parts which had to develop new heads retained their juvenility. This result indicates that whereas daughter individuals that retain old cells eventually die, daughter individuals that have to replace cells remain young. It is interesting to note that rotifers, which are also clonally propagated, though not by the process of fission, undergo no cell replacement during their life-span, and on the basis of Sonneborn's result individuals could be said to age and die out as a consequence.

Maynard-Smith has postulated a "Threshold theory of Ageing", based on the results he obtained from the survival of fruit flies cultured at different temperatures for different parts of their life-spans. It was found that flies cultured first at a low temperature and then transferred to a high temperature showed a reduction in life expectancy at the high temperature by one day for every day spent at the lower temperature. However flies cultured first at a high temperature and then at a lower temperature died at the same time as flies kept at a lower temperature throughout life, unless the time at the high temperature had brought them to within a certain critical period of expected death. If this had happened

then removal to a low temperature conferred no extension of the life-span over that of the peers that remained at a high temperature. From this kind of result he concluded that when a certain level of vitality which he called the threshold had been reached the animals started to die irreversibly at a rate independent of temperature, but the time taken to reach this threshold varied inversely with the culture temperature. It is not possible to directly relate the temperature dependent threshold of Drosophila sp. to the experiments performed on the rotifer Mytilina brevispina var redunca in this study, however the concept of a threshold of vitality may be applicable in so far as there could be a threshold value of calcium content which inevitably leads to death. In the case of rotifers treated throughout life with chelating agents, the extension of their lives may have been due to an extension in the time needed to reach the threshold, but the treatment was presumably not effective in preventing it being reached at all. The culture transfer experiments carried out at day 3 may not have shown the sort of effect predicted by Maynard-Smith's results as they occurred too early in life (i.e. during the ageing phase). However, at a later time in life (i.e. during the dying phase), rotifers transferred from Low to High calcium medium may have shown a reduction in longevity that was proportional to the time spent in the Low calcium medium, while rotifers, unless they were within a critical period of expected death transferred from High to Low calcium medium, may have shown a similar longevity to rotifers cultured throughout life in Low calcium medium. If the rotifers that were transferred from High to Low calcium medium were within a critical period of imminent death, the individuals transferred would not have been expected to show any extension in life-span compared with the peers maintained throughout life on High calcium medium. These speculated results could only be verified by further serial transfers performed on separate groups of rotifers every day after the third day of life and similar transfers between High and Low temperatures could also be conducted.

Bjorskten in 1946 stressed the importance of intracellular cross-linking between proteins as a possible cause of ageing. In support of such a theory is the well known fact that fats are particularly liable to form cross linkages, a phenomenon that in the long term could be hazardous to biological systems. I have attempted to treat rotifers with the anti-crosslinking agent b aminopropionitrile to see whether life-span could be significantly prolonged. Unfortunately, this chemical appeared to be extremely toxic in the short term, and further experiments would be required to find a sub toxic, yet effective concentration of this substance or other anti-crosslinking agents non toxic to rotifers.

From a brief consideration of a few of the more pertinent theories of ageing, it would appear that the results of this present study are in complete agreement with the Lansing theory of ageing that proposes a detrimental accumulation of calcium after the completion of growth in size. Part 2 of this study deals with the effects of antioxidation and dietary restriction on rotifers, and the relationships between these theories and rotiferan ageing are discussed in the Part 2 Discussion.

PART 2

INTRODUCTION TO ANTIOXIDATION EXPERIMENT

Two approaches to ageing that receive much attention in the field of gerontology are those of Autoxidation and Dietary Restriction. Both of these approaches have successfully prolonged life in several inbred species of rats, and in the case of autoxidation have so far not been applied to the Rotifera.

Much importance is attached to the action of free-radicals in theories of Ageing based on autoxidation. These constitute chemically unstable molecular species that owe their instability to the presence of unpaired electrons. They may be formed during the course of many biological reactions as unstable intermediates or by the exogenous effects of such phenomena as ionising radiation. The radolytic decomposition of water for instance produces the hydroxy free radical that can attack D.N.A. nucleotides such as thymine, guanine, and cytosine. Although the attachment of the free radical to D.N.A. stabilizes the free radical, D.N.A. itself is rendered unstable and may react with other D.N.A. molecules destroying particular coding regions. D.N.A. is a well known target of free radical attack, peroxy free radicals and singlet oxygen dimers are two further groups of free radicals that can produce serious biological damage in this molecule. However, there are substances known as free radical scavengers or antioxidants that are capable of stabilizing free radicals, thereby rendering them unreactive chemical species with regard to such targets as D.N.A., Lipids, R.N.A. synthetase, etc. The free radical antioxidant complex does not, like the free radical D.N.A. complex, become an unstable chemical species capable of further molecular attack. In this present study three free radical scavenging antioxidants have been applied to three populations of rotifers to ascertain whether or not they could produce increased life as a result of reducing free radical attack.

MATERIALS AND METHODS

ANTIOXIDATION EXPERIMENT

The four groups of 30 rotifers employed in the experiment were cultured under identical conditions to those described on Page 36 . The first group of 30 was adopted as a Control group and was cultured throughout its life period in Knop's Control medium, total salt concentration 0.04% (see Table 4). The remaining 3 groups were cultured individually on Knop's Control medium that had been supplemented in each case with one of the following three water-soluble antioxidants: cysteine hydrochloride, sodium hypophosphite and butylated hydroxy-toluene, to give a total salt concentration in the Control medium of 0.05%. This value lay well within the predetermined limits of total salt tolerance for this species. All individual cultures were changed daily, and the number of survivors and number of eggs produced were recorded on each day throughout the life period of each group.

RESULTS

ANTIOXIDATION EXPERIMENT

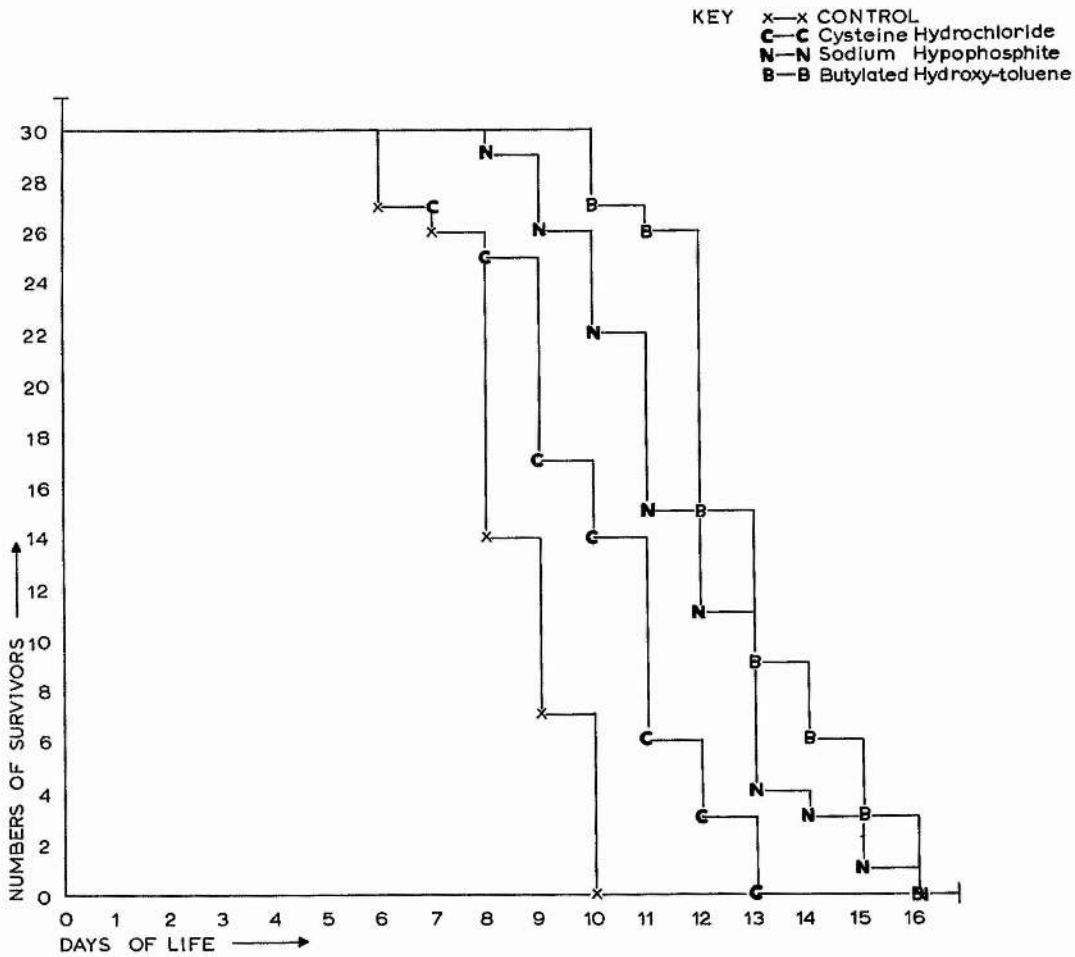
The first mortalities in the untreated Control group occurred at day 6, three days after the period of growth and maturation (see Graph 19). Thereafter, a consistent marked decline in the number of survivors began at day 8, when 12 rotifers (40% of the original population number) died. This decline continued on days 9 and 10 with seven individuals (about 23% of the original population number) dying on each day. No survivors were recorded in the untreated Control group after day 10, and the mean longevity value of the population was 8.5 days.

The first mortalities in the cysteine hydrochloride treated group occurred at day 7 (one day after those of the untreated Control group) when three rotifers died. On day 10 when the last of the

TABLE 14

ANTIOXIDATION EXPERIMENT

POPULATION	MEAN LONGEVITY	INCREASE IN LIFE EXPECTANCY	TOTAL EGGS
CONTROLS	8.5 days S.E. ± 0.2 days		139
CYSTEINE HYDROCHLORIDE	10.4 days S.E. ± 0.3 days	22.3%	110
SODIUM HYPOPHOSPHITE	11.7 days S.E. ± 0.2 days	37.6%	253
BUTYLATED HYDROXY TOLUENE	12.9 days S.E. ± 0.2 days	52.0%	284

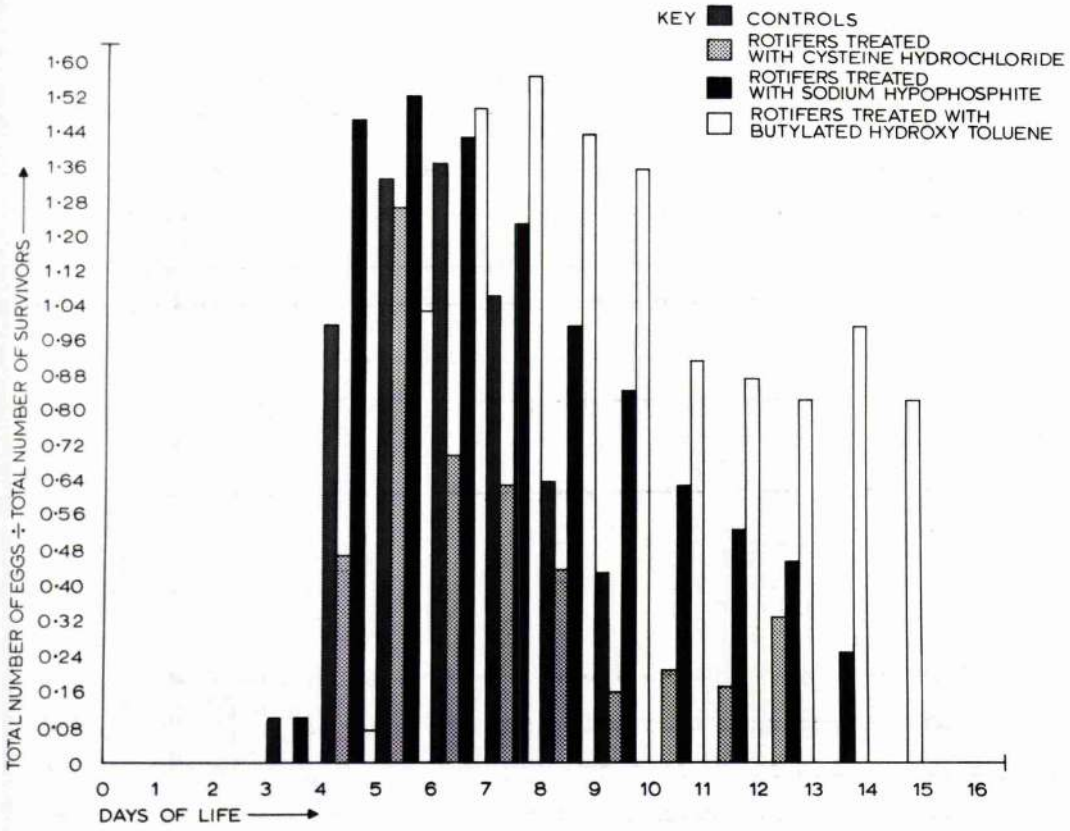

KEY x—x CONTROL
C—C Cysteine Hydrochloride
N—N Sodium Hypophosphite
B—B Butylated Hydroxy-toluene
NUMBERS OF SURVIVORS
DAYS OF LIFE
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Graph 19

untreated Control rotifers expired, 14 rotifers, corresponding to about 47% of the original population number, still survived in the group, eight of the previous mortalities having occurred on day 9. On the following day, day 11, eight of the surviving population at day 10 were recorded dead, the six survivors on this day dying at the rate of three rotifers each day on the following two days. No rotifer in the group survived beyond day 13 and the mean longevity value recorded was 10.4 days, representing a 22.3% increase in life expectancy over the untreated Controls (see Table 14).

A first single mortality in the sodium hypophosphite treated group occurred at day 8, two days after the first mortalities recorded in the untreated Controls and one day after those in the cysteine hydrochloride treated group. At day 10 when the entire untreated Control population had expired and only 14 rotifers survived in the cysteine hydrochloride group, 22 rotifers (about 73% of the original population number) that received treatment with sodium hypophosphite were still alive, all except one of the deaths having been evenly distributed between days 9 and 10. On day 13 when the last survivors in the cysteine hydrochloride group expired, only four rotifers in the sodium hypophosphite group were still alive, this being due to the particularly marked decline in population number on days 11 and 13, seven rotifers dying on each day. Of the four survivors at day 13 only one of them died the following day, two individuals surviving till day 15, and one rotifer, the last member of the group surviving till day 16. The mean longevity value of the sodium hypophosphite group was 11.7 days this value representing a 37.6% increase in longevity over the untreated Controls and a 5.3% increase in longevity over the cysteine hydrochloride treated population (see Table 14).

The first mortalities in the B.H.T. treated group occurred on day 11, one day after the entire untreated Control population had expired, and 4 and 3 days respectively after the first deaths in the


KEY
CONTROLS
ROTIFERS TREATED WITH CYSTEINE HYDROCHLORIDE
ROTIFERS TREATED WITH SODIUM HYPOPHOSPHITE
ROTIFERS TREATED WITH BUTYLATED HYDROXY TOLUENE
TOTAL NUMBER OF EGGS ÷ TOTAL NUMBER OF SURVIVORS
1·60
1·52
1·44
1·36
1·28
1·20
1·12
1·04
0·96
0·88
0·80
0·72
0·64
0·56
0·48
0·40
0·32
0·24
0·16
0·08
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS OF LIFE

Histogram 7

cysteine hydrochloride and sodium hypophosphite groups. However, a particularly marked decline in the population number occurred at day 12 when 11 rotifers (about 37% of the original population) expired. A further six deaths were recorded the following day, day 13, so that when the entire cysteine hydrochloride treated group had expired, and four rotifers survived in the sodium hypophosphite treated population, only nine rotifers were still alive in the B.H.T. treated group. These nine rotifers died at the rate of three rotifers a day on each of the following three days, days 14, 15 and 16, consequently none of the B.H.T. group outlived the sodium hypophosphite group which also expired at day 16. However, the B.H.T. treated group displayed the highest average longevity value of 12.9 days of any of the other experimental populations. This longevity value represented a 52.0% increase in life expectancy over the Controls, and 29.7% and 24.4% life expectancy increases over the cysteine hydrochloride and sodium hypophosphite treated populations respectively (see Table 14).

The egg production in the untreated Control group began at day 3 when only three eggs were laid (see Histogram 7). At day 4 the entire population of 30 laid one egg and even higher levels of egg laying than this were recorded up till day 8, when only just over half the survivors laid one egg. The last recorded eggs in the Control group were laid by just under half the number of survivors at day 9.

Of all the experimental populations only the sodium hypophosphite group began to lay eggs on day 3, the same day as the untreated Controls. The cysteine hydrochloride and B.H.T. treated populations did not begin egg-laying till day 4, and even on this day less than a quarter of the B.H.T. group laid a single egg. However, both the groups that showed a delay in the onset of egg-laying showed levels of egg-laying that exceeded one egg per individual on the following day. The cysteine hydrochloride group showed a marked decline in their egg production at day 6, a decline that preceded any decline in the Controls by one day, and which continued up till day 12 when a slight final increase in egg-laying rate was recorded. By way of contrast the sodium hypophosphite and

B.H.T. treated groups maintained a rate of egg-laying that exceeded one egg per survivor up till days 8 and 10 respectively, both declines occurring after a decline in the Controls.

After day 8 the rate of egg production in the sodium hypophosphite treated group showed a gradual and uniform decline up till day 13, when the last eggs were laid (4 days after the final eggs were recorded for the untreated Controls). The B.H.T. treated group showed the highest rate of egg production in any of the groups after day 7, with never less than 4/5ths the survivors laying a single egg up till the final day of egg-production at day 15, 6 days after the final eggs were produced by the untreated Controls.

PRELIMINARY DISCUSSION

ANTIOXIDATION EXPERIMENT

All three free radical scavenging antioxidants produced significant increases in life expectancy when added as a 0.01% supplement in the total salt concentration of Knop's Control medium. Life expectancy compared to the untreated Control group was greatest in the B.H.T. treated group, 52.0%, intermediate in the sodium hypophosphite treated group, 37.6%, and least in the cysteine hydrochloride treated group, 22.3%. The forms of the survival graphs in the case of the experimentally treated populations did not approach the rectangular shape, indicating senescence with almost simultaneous death, as closely as the Control group. Instead a flattening out of the survival graph at the end of the life period, particularly in the case of the sodium hypophosphite group, was evident. These differences in mortality rate at the end of an artificially prolonged life may have been a reflection of a lack of physiological uniformity between senescent rotifers in experimentally treated populations, or of different sensitivites to toxic effects of the antioxidants.

In all cases longevity increases were accompanied by an increase in the length of the reproductive period. The B.H.T. and sodium hypophosphite groups displayed reproductive periods that were four days longer than that recorded for the untreated Controls, while the cysteine hydrochloride group extended its reproductive period by only two days. It is interesting to note that with the exception of the sodium hypophosphite group the antioxidant treated populations showed a delay in the onset of egg laying, and this was particularly marked in the case of the B.H.T. group which showed the greatest increase in life expectancy. However, whereas the delay in the start of the reproductive period in the B.H.T. treated group was followed by a consistently high rate of egg-production throughout life, the cysteine hydrochloride group showed a decline in its level of egg production that preceded a decline in the untreated Controls. Even though the duration of the reproductive period in the cysteine hydrochloride group was extended by two days the total number of eggs laid during its life period was 29 eggs less than the total recorded for the Control group (see Table 14). On the basis of Lansing's 1942 argument that egg production was an index of the level of metabolism, the cysteine hydrochloride population displayed a level of metabolism that was lower than that of the Controls during most of the corresponding part of its life period, and this may have indicated that the increased life expectancy recorded for this group was the result of a lowered level of metabolism rather than a true delay in the ageing process.

MATERIALS AND METHODS

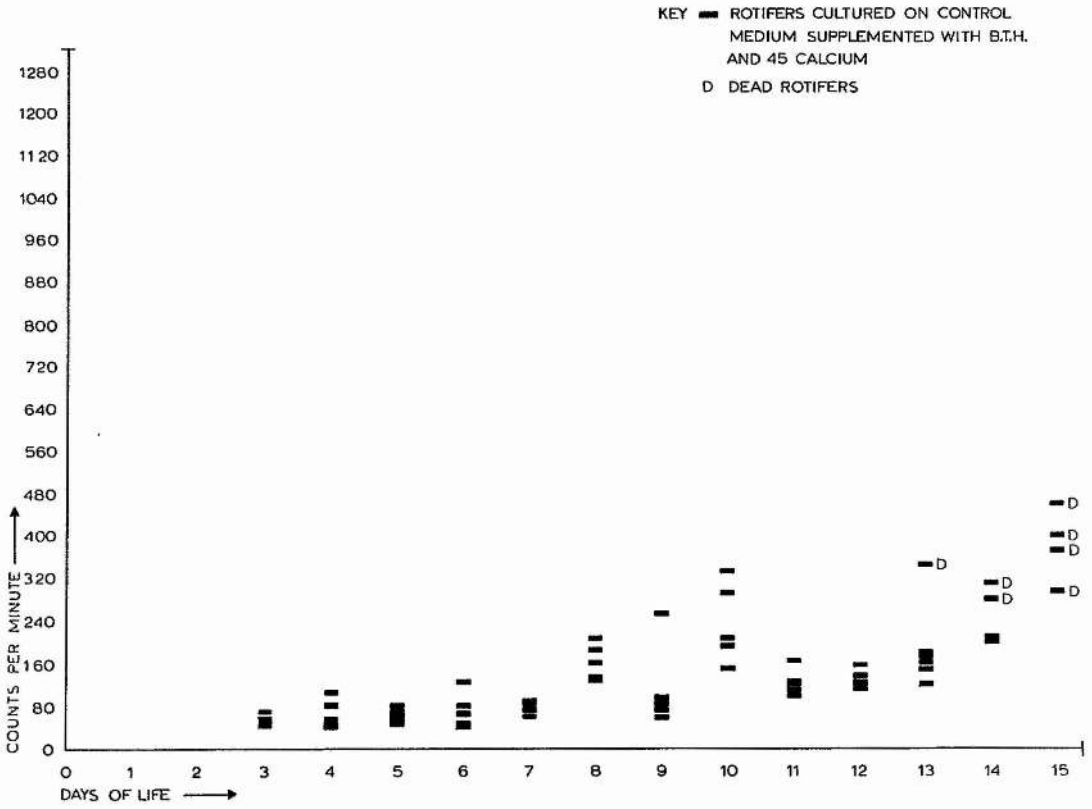
RADIOTRACER EXPERIMENT 2

Two groups of 30 rotifers were individually cultured under strictly aseptic conditions on Knop's Control media. The first group was cultured on Control medium which contained the radionuclide 45 calcium, while the second group was cultured on Control medium containing the same quantity of radionuclide and in

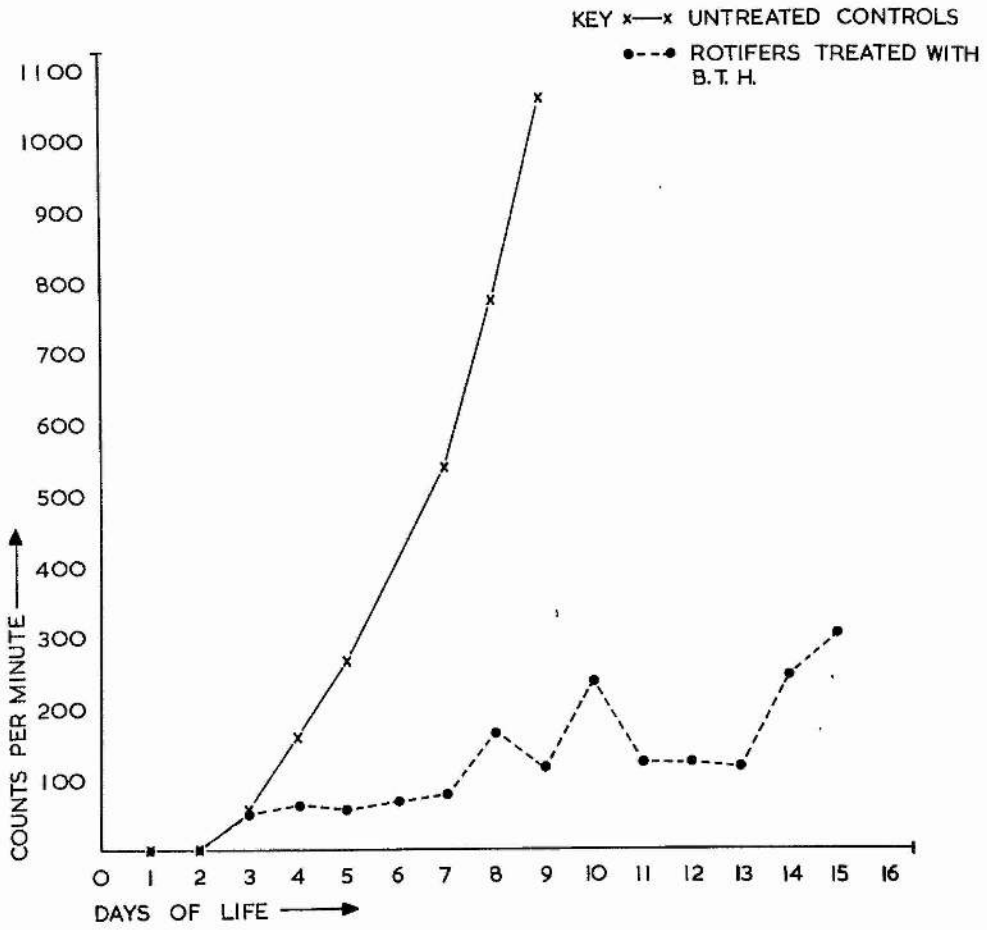
TABLE 15

RADIOTRACER EXPERIMENT 2

POPL.	MEAN OF 5 ROTIFERS COUNTS PER MINUTE/DAY BELOW WTS X 10^{-12} gm 45 Ca														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CONTROLS			56 4.59	156 12.75	272 22.43	330 27.27	539 44.13	774 63.39	1060 86.82						
B.H.T.			50 3.9	65 5.31	60 4.89	70 5.70	80 6.54	165 13.47	113 9.21	235 19.17	120 9.78	120 9.39	115 9.39	240 19.59	300 24.48


KEY ROTIFERS CULTURED ON CONTROL MEDIUM SUPPLEMENTED WITH B.T.H. AND 45 CALCIUM
D DEAD ROTIFERS
COUNTS PER MINUTE
1280
1200
1120
1040
960
880
800
720
640
560
480
400
320
240
160
80
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
DAYS OF LIFE
D
D
D
D
D
D
D

Graph 20


KEY x—x UNTREATED CONTROLS
●---● ROTIFERS TREATED WITH B.T.H.
1100
1000
900
800
700
600
500
400
300
200
100
COUNTS PER MINUTE
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS OF LIFE

Graph 21

addition 0.01% of the antioxidant B.H.T., bringing the total salt concentration of the modified Knop's medium to 0.05%. All radioactive culture droplets were maintained on plastic depression slides to prevent exchanges occurring between the 45 calcium of the culture medium and the 40 calcium silicates of glass. The algal supplement was monitored at the same level as before in all culture droplets, and all cultures were again changed daily. On every day during the course of the experiment at least five rotifers were removed from each of the two experimental populations and washed in three separate 3ml volumes of 40 calcium Knop's Control medium before being transferred to a millipore filter disc. All samples were pooled and counted on the same day by scintillation counting technique at the end of the experiment, thereby avoiding the correction of individual samples for different decay factors.

RESULTS

RADIOTRACER EXPERIMENT 2

The mean 45 calcium intake in the Control and antioxidant treated groups was not significantly different on day 3, when the first counts were obtained in the rotifers sampled (see Graphs 20, 21 and Table 15). Thereafter, the antioxidant treated group accumulated 45 calcium at a much slower rate than the untreated Controls, with less than 100 cpm being recorded in all antioxidant samples up till day 8, by way of contrast the Control group accumulated over 100 cpm on the day following the first recorded count at day 3. At day 9 when the rate of 45 calcium accumulation in the Controls was at a maximum producing a final count of 1060 cpm (86.82×10^{-12}gms), the mean count in the antioxidant treated samples had declined from the increased count obtained the previous day to 113 cpm (9.21×10^{-12}gms). On day 10 the antioxidant treated samples showed an increased rate of 45 calcium intake that again was not sustained in the samples counted the following day, indeed it was not until day 13 of life that a total 45 calcium count was sustained

for two consecutive days in the samples selected for counting. The final count obtained at day 15 of 300 cpm (24.48×10^{-12} gms) was 760 cpm (20.74×10^{-12} gms) below that of the final count obtained for the untreated Control samples.

PRELIMINARY DISCUSSION

RADIOTRACER EXPERIMENT 2

The B.H.T. treated population in Radiotracer Experiment 2 was cultured under identical conditions to the B.H.T. treated group in the Antioxidation Experiment, with the notable exception that the Knop's Control culture medium contained the radionuclide 45 calcium. The five individuals sampled daily from the radioactive antioxidant treated group reflected a much lower rate of 45 calcium accumulation than was observed for the Control samples, and this accumulation occurred not in the uniformly cumulative pattern of the Control group, but sporadically at various points in the life period (see Graph 21).

INTRODUCTION TO DIETARY RESTRICTION EXPERIMENT

The 1965 study of Fanestil and Barrows performed on the rotifer Philodina acuticornis constitutes the major dietary restriction study performed on rotifers to date, and is broadly related to previous dietary restriction studies performed by Lynch and Smith in 1931. Three nutrient regimes were set up in the 1965 study, each of them being based on boiled filtered lake water. The first regime contained an uncounted number of Chlamydomonas cells, while the second and third regimes contained no algal supplement. Individuals cultured on regimes 1 and 2 were transferred to fresh cultures daily, while those on regimen 3 were only transferred to fresh cultures on Mondays, Wednesdays and Fridays of each week. The results of the experiment indicated that mean life-span was longest for regimen 3 rotifers (54.7 days), shortest for regimen 1 rotifers (34.0 days), and intermediate for regimen 2 rotifers (45.3 days),

i.e. mean longevity was inversely related to the food level in each of the three nutritional groups. It should be mentioned that this work has been criticised by King et al on the grounds that during the period of storage of the boiled filtered lake water contamination with bacteria could have occurred, thereby producing an additional and uncontrolled food source in each of the three media (particularly as it is well established that the Order Bdelloidea of which Philodina acuticornis is a member, responds favourably to bacterial culture regimes, Meadow and Barrows 1969). It is also unfortunate that no information concerning growth rate in each of the three populations was given, which would have been particularly relevant to earlier dietary restriction studies performed by McCay and Cromwell 1934, and McCay, Cromwell and Maynard 1935.

The nutritional study performed by McCay in 1928 on brook trout, in which a 10% restricted protein diet produced a failure to grow and a resultant doubled life span, led this author to perform a further nutritional study in 1934 on three groups of white rats. The first group was reared on a full diet and grew and matured rapidly, the second group was reared on a diet reduced in total calorific value though not in the quality of essential nutrients, and grew and matured slowly, and the third group was grown on the full diet for two weeks after weaning and then forced to mature slowly on the restricted diet of the second group. It was found that the rapidly grown male rats had a median life span of 522 days compared with longer median life span periods of 797 and 919 days in the groups that were respectively slowly grown and slowly matured on the restricted diets (data from Table 1 McCay and Cromwell 1934). It was this sort of result together with the dietary restriction results of Fanestil and Barrows 1965, that led to the dietary restriction experiments performed in this study.

MATERIALS AND METHODS

DIETARY RESTRICTION EXPERIMENT

Four groups of 30 rotifers were employed in the experiment. The first group of 30 was cultured under identical conditions to those described for the untreated Control group in the Ion Ratio Experiment. The other three groups were cultured on the Control, Low or High calcium medium (see Table 4), all 0.02 ml culture droplets containing an algal component reduced from 5000 to 1900 cells per culture, approximately 38% of the normal concentration employed. Algal levels were monitored to an accuracy of $\pm$ 50 cells per culture by means of a micro-nephelometer in conjunction with a haemocytometer. The culture droplets were renewed every day, and daily observations were made of the numbers of survivors and the number of eggs laid in each group. Although complete growth curves were not plotted for each of the four groups, growth measurements between widest transverse and longitudinal body axes were carried out on each experimental rotifer by means of an eye-piece graticule, beginning early on day 3, in order to ascertain whether the growth period exceeded the usual three days in any of the experimental populations, and whether the average ultimate size in each group differed from that of the untreated Controls.

RESULTS

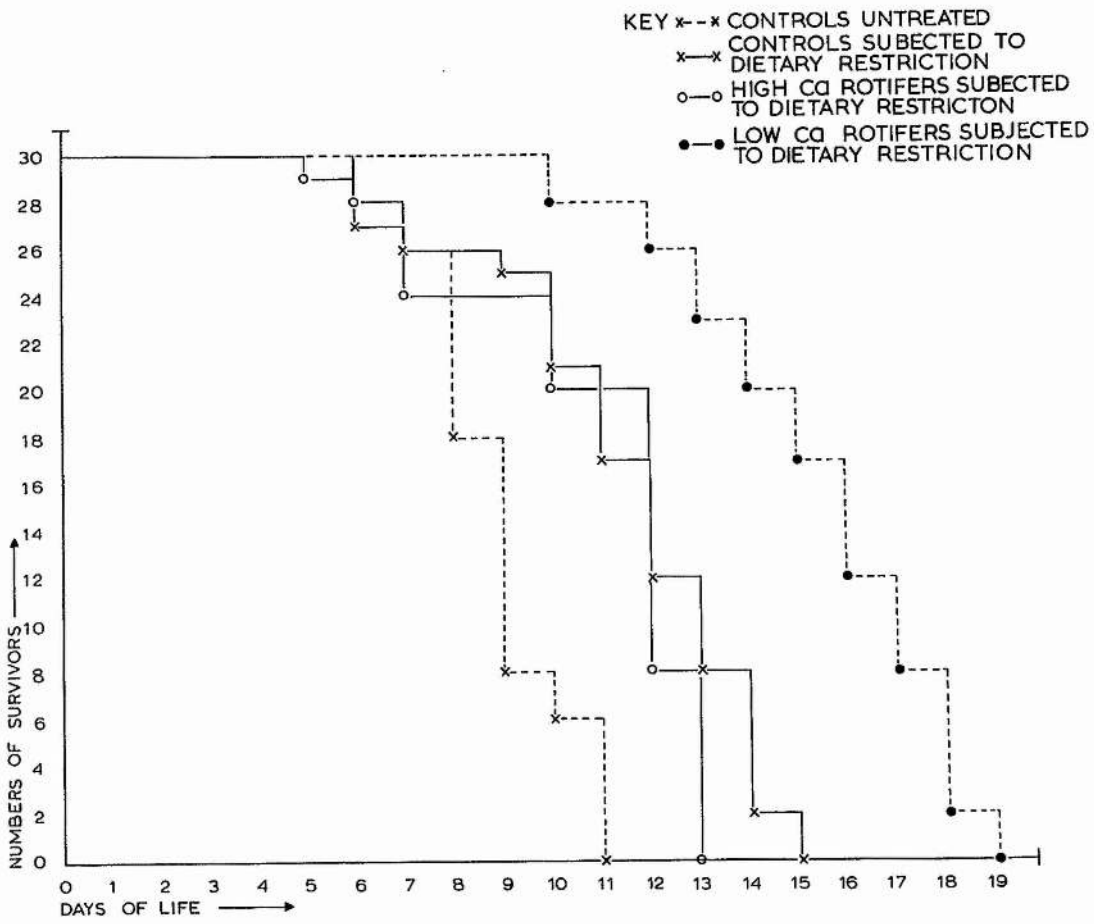
DIETARY RESTRICTION EXPERIMENT

The first mortalities in the untreated Controls occurred at day 6, 2 days after the period of growth and maturation (see Graph 22). Thereafter, a marked reduction in the number of survivors began at day 8 when eight rotifers, corresponding to about 27% of the original population number, died. This decline in numbers continued at a slightly faster rate the following day, day 9, when ten individuals expired, though at day 11 this death rate was markedly reduced to the rate of two mortalities on this day. The final six deaths that

TABLE 16

DIETARY RESTRICTION EXPERIMENT

POPULATION	MEAN LONGEVITY	INCREASE IN LIFE EXPECTANCY	TOTAL EGGS
UNTREATED CONTROLS	8.8 days S.E. ± 0.2 days	-	146
TREATED CONTROLS	11.8 days S.E. ± 0.2 days	34.1%	242
TREATED HIGHS	10.9 days S.E. ± 0.3 days	23.8%	212
TREATED LOWS	15.8 days S.E. ± 0.3 days	79.5%	368


KEY x--x CONTROLS UNTREATED
x—x CONTROLS SUBECTED TO DIETARY RESTRICTION
o—o HIGH Ca ROTIFERS SUBECTED TO DIETARY RESTRICTON
•—• LOW Ca ROTIFERS SUBJECTED TO DIETARY RESTRICTION
NUMBERS OF SURVIVORS
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
DAYS OF LIFE

Graph 22

led to the extinction of the untreated Control group occurred at day 11.

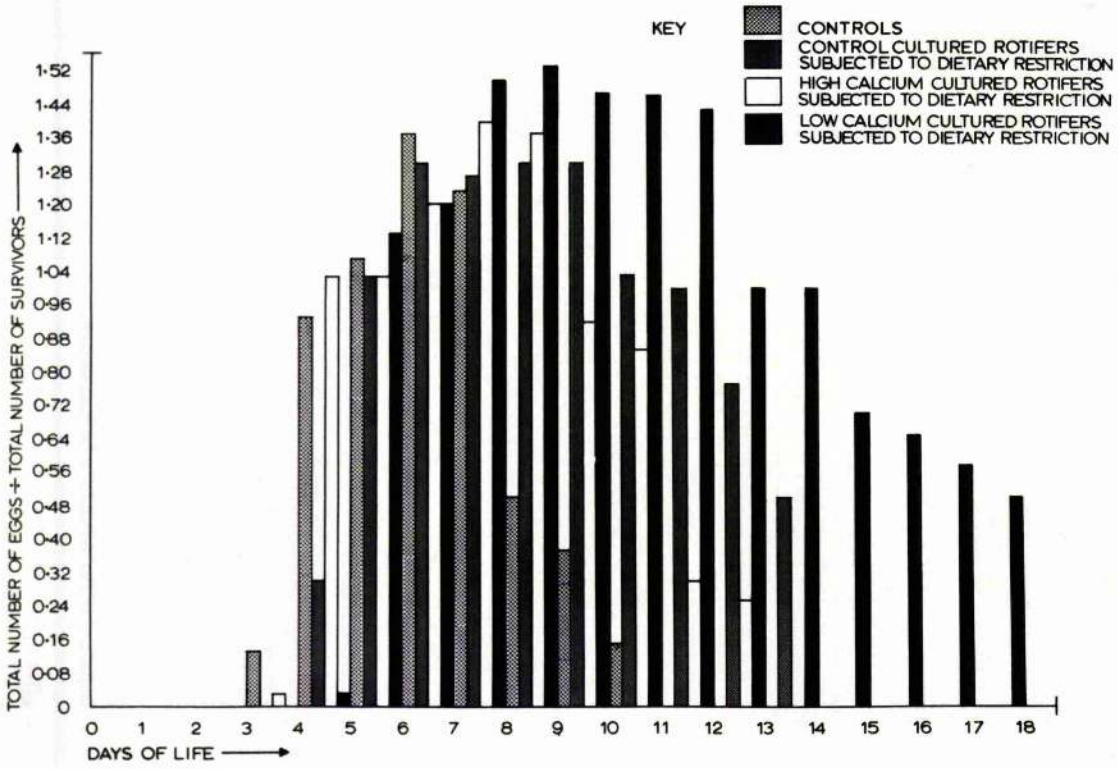
The first mortalities in the nutritionally restricted Control group occurred at day 9 (3 days after those of the untreated group), when five rotifers died. Thereafter, up till day 14 rotifers of the nutritionally restricted group died at the rate of four rotifers each day, with the exception of day 12 when five deaths were recorded, this consistent death rate giving rise to a population of 17 on the day of extinction of the untreated Control colony, day 11. On day 14 six rotifers died in the nutritionally restricted group, the remaining two survivors dying the following day, day 15. The mean longevity of the nutritionally restricted Control group was 11.8 days, corresponding to a 34.1% increase in life expectancy over the untreated Controls (see Table 16).

A single first mortality in the High calcium group subjected to dietary restriction occurred one day before the first mortalities in the untreated Controls, at day 5. In the following 2 day period five rotifers died, and on day 7 the 24 rotifers surviving in the group numbered two less than the total number of untreated Control survivors on this day. However, by way of contrast with the untreated Controls no further deaths were recorded in the nutritionally restricted High calcium group until day 10, when four rotifers died. On day 11, the day of extinction of the untreated Control colony, no mortalities were recorded in the nutritionally restricted High calcium group, whose total population still numbered 20 rotifers. These rotifers all died in the following two days, days 12 and 13, with the particularly high number of 12 mortalities (40% of the original population number) being recorded on day 12. The mean longevity value of the nutritionally restricted High calcium group was 10.9 days, corresponding to a 23.8% increase in life expectancy over the untreated Controls and a 7.6% reduction in life expectancy compared with the nutritionally restricted Control group (see Table 16).

The first mortalities in the Low calcium nutritionally restricted group occurred at day 10, when two rotifers died. No deaths were recorded at day 11, when the untreated Control colony became extinct, giving a total population at this time of 28 rotifers. After day 12, when two deaths were recorded, a consistently low mortality rate of three deaths per day was recorded up till day 16, when five individuals died. The population on days 13 and 15, the days of extinction of the High calcium and untreated Control colonies, numbered 23 and 17 individuals on each day respectively. Of the twelve survivors at day 16, four died the following day and six died at day 18. The last two survivors in the group died the following day, day 19. The mean longevity value of the nutritionally restricted Low calcium group was 15.8 days, corresponding to a 79.5% increase in life expectancy over the untreated Controls and a 33.9% increase over the nutritionally restricted Control group (see Table 16).

The growth measurements of 30 individuals in each group that were begun early on day 3 showed no delay in the termination of the growth period in the experimental groups as compared with the untreated Controls. The ultimate size attained in all groups corresponded closely to that revealed by populations cultured on the same media but a full diet in the Ion Ratio Experiment, with the Low calcium groups showing a similar reduction in size, and the High calcium groups showing a similar increase in size, compared to the Control groups whose final size all corollated closely.

Egg-laying began in the untreated Control group on day 3, when four rotifers each laid an egg (see Histogram 8). Each member of the surviving population laid at least one egg a day up till day 8, when a marked decline in egg production was noted, only half the number of survivors laying a single egg on this day. This decline in egg production continued till day 10, when the last egg was laid by one of the surviving members of the group. The overall length of the reproductive period in this group was 8 days.


KEY
CONTROLS
CONTROL CULTURED ROTIFERS SUBJECTED TO DIETARY RESTRICTION
HIGH CALCIUM CULTURED ROTIFERS SUBJECTED TO DIETARY RESTRICTION
LOW CALCIUM CULTURED ROTIFERS SUBJECTED TO DIETARY RESTRICTION
TOTAL NUMBER OF EGGS + TOTAL NUMBER OF SURVIVORS
1·52
1·44
1·36
1·28
1·20
1·12
1·04
0·96
0·88
0·80
0·72
0·64
0·56
0·48
0·40
0·32
0·24
0·16
0·08
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
DAYS OF LIFE

Histogram 8

The nutritionally restricted Control group did not begin egg-laying until day 4 (one day after the first eggs were recorded for the untreated Controls), when nine rotifers each laid an egg. The level of egg-laying then exceeded the rate of one egg per day per survivor up till day 12, when only three quarters of the surviving population laid a single egg. This decline in egg production which followed a decline in the untreated Controls recorded 4 days earlier, continued gradually until day 13, when the last eggs were laid by the group. The overall length of the reproductive period of 10 days was 2 days longer than that recorded for the untreated Controls.

The High calcium group was the only nutritionally restricted group to begin egg-laying on the same day as the untreated Controls, day 3, albeit only a single egg was laid on this day. Thereafter, the rate of egg production never fell below the rate of 1 egg per day per individual until day 9, when two of the population number surviving at this time failed to produce an egg. This decline in egg-laying, which occurred one day after a more marked decline in egg production in the untreated Control group, continued until day 12 when the last eggs were laid by two of the surviving members of the group. The overall length of the reproductive period was 10 days, 2 days longer than that of the untreated Controls.

The nutritionally restricted Low calcium group, the longest lived group in the experiment, showed the greatest delay in the onset of egg-laying till day 4, when only one egg was laid. However, the following day, day 5, at least one egg was laid by the entire population in the group. The rate of egg-laying never fell below the rate of one egg per survivor per day until day 14, when just under three quarters of the surviving population at the time laid a single egg. Egg-laying did not cease in the group until day 17, when still at least half the number of survivors at that time laid a single egg. The overall reproductive period of 13 days was five days longer than that recorded for the untreated Control group.

TABLE 17

EXPERIMENT TITLE	CULTURE MEDIUM	LONGEVITY VALUE	CHANGE OF LONGEVITY VALUE	
			(a) BETWEEN CORRESPONDING GROUPS IN THE 2 EXPERIMENTS	(b) RELATIVE TO EACH EXPERIMENTAL CONTROL
ION RATIO EXPERIMENT	HIGH CALCIUM	7.6 days $\pm$ 0.2 days	-	12.6% reduction
	CONTROL CALCIUM	8.7 days $\pm$ 0.2 days	-	-
	LOW CALCIUM	12.2 days $\pm$ 0.3 days	-	40.2% increase
DIETARY RESTRICTION EXPERIMENT	HIGH CALCIUM	10.9 days $\pm$ 0.3 days	43.4% increase	7.6% reduction
	CONTROL CALCIUM	11.8 days $\pm$ 0.2 days	35.6% increase	-
	LOW CALCIUM	15.8 days $\pm$ 0.3 days	29.5% increase	33.9% increase

Ion Ratio Experiment data was compared with the Dietary Restriction Experiment data as the untreated Control group of the latter experiment exhibited a mean longevity value of 8.8 days $\pm$ 0.2 days which did not significantly differ from the value obtained for the corresponding group in the Ion Ratio Experiment.

All experimental populations showed good egg production maxima during their reproductive periods. However the nutritionally restricted Low calcium group showed the highest maxima of all groups, and maintained the highest rate of egg-laying over the longest period.

PRELIMINARY DISCUSSION

DIETARY RESTRICTION EXPERIMENT

All the populations subjected to dietary restriction showed a prolonged mean longevity compared to the Control group cultured on a full diet (see Table 17). It is interesting to note that in the Ion Ratio Experiment the High calcium population showed a 12.6% reduction in life expectancy compared with the Controls, while the Low calcium population showed a 40.2% increase in longevity compared with the same Control group. The results for the corresponding populations cultured on a reduced diet show approximately the same differences in longevity between groups, though the overall mean longevity value of each group is increased. The nutritionally restricted High calcium group showed a 7.6% reduction in life expectancy, (5.0% less than the life expectancy reduction shown by the High calcium group in the Ion Ratio Experiment) with respect to the nutritionally restricted Controls, while the Low calcium group showed a 33.9% increase in life expectancy (6.3% below the increase in life expectancy of the corresponding Low calcium group in the Ion Ratio Experiment) with respect to the same nutritionally restricted Controls. The overall increases in life expectancy shown by the nutritionally restricted groups with respect to the same groups cultured on a full diet in the Ion Ratio Experiment were a 35.6% increase in life expectancy for the nutritionally restricted Controls over the Controls cultured on a full diet, a 43.4% increase in life expectancy for the nutritionally restricted High calcium group over the corresponding group cultured on a full diet, and a 29.5% increase in life expectancy for the nutritionally restricted Low calcium group over the same group grown on a full dietary regime.

The High calcium group would appear to show the greatest relative increase in life expectancy as a result of nutritionally restricted culture. It is also interesting to note that the nutritionally restricted High calcium group showed a marked incidence of early mortalities with respect to the untreated Controls, which could indicate that the reduced nutritional level deviated from the optimum for the early development of some members of the group.

It is particularly relevant to the nutritional studies of McCay (McCay 1934 and 1935) that growth in the nutritionally restricted groups ceased at the same time as growth in the Controls that had been cultured on a full diet. The differences in ultimate size attained, closely correlated with those shown by corresponding populations cultured on a full diet. Although none of the nutritionally restricted populations showed a reduction in overall rate of egg-laying compared with corresponding populations cultured on a full diet, both the Low calcium and Control dietary restricted groups showed a delayed onset of egg-laying that may have reflected a slight delay in maturation. It should be noted however, that a delay in the onset of egg-laying was also observed in the Low calcium group cultured on a full dietary regime.

MATERIALS AND METHODS

ALGAL CALCIUM UPTAKE EXPERIMENT

An experiment was designed to assess the 45 calcium intake of the rotifers through the consumption of algae in normal experimental diets. To do this an equivalent number of 5000 algal cells were exposed to 45 calcium in each of ten 0.02 ml droplets of Knop's Control medium supplemented with the same concentration of radionuclide, over a 24 hour period, the period of time for which cultures were normally established before being renewed. To prevent exchanges occurring between the 45 calcium of the Control culture medium and the 40 calcium silicates of glass, all culture droplets were maintained on perspex slides. After the 24 hour exposure period, the entire contents of each radioactive culture were

transferred to a Millipore filter disc mounted on a holder, the perspex slides being rinsed several times with Knop's Control 40 calcium medium to ensure all algal cells had been transferred to the filter disc. Twenty millilitres of Knop's Control 40 calcium medium were then washed through each filter disc to remove as much of the externally bound 45 calcium as possible, before each disc was counted by scintillation counting technique. Any background contamination that remained on the sample discs after the washing procedure, was estimated by establishing ten 0.02 ml droplets of Knop's Control 45 calcium medium without algae, for 24 hours on the perspex slides. These droplets were then transferred individually to 10 filter discs (again the slides were rinsed with Knop's Control 40 calcium medium) and after a 20 ml volume of Knop's Control 40 calcium medium was washed through each disc, all the discs were collected and counted by scintillation counting technique on the same day as the other samples, in order to avoid having to correct for individual sample decay factors. The mean count of the sample blanks was subtracted from the mean count obtained from the algal samples, all samples being counted ten times.

In order to obtain an approximate value for the number of algae ingested in one day by a rotifer, observations lasting one hour were carried out on ten 4 day old rotifers every 4 hours beginning at 8 a.m. and ending at 11 p.m. The rotifers were each cultured in 0.02 ml droplets of Knop's Control medium containing the normally adopted level of algae corresponding to 5000 cells per culture. Care was taken to count only the algal cells actually ingested (ingestion was always accompanied by a sudden contraction of corona and mastax) and not the cells rejected during normal sampling procedures.

RESULTS

ALGAL CALCIUM UPTAKE EXPERIMENT

The results are summarised in Table 18. They indicate that a count of 1033 cpm (84.6×10^{-12} gms) was obtained from 5000 cells cultured in 0.02 mls of Knop's Control 45 calcium medium for 24 hours.

TABLE 18

ALGAL CALCIUM UPTAKE EXPERIMENT

MEAN BLANK (mb) cpm	MEAN FOOD (MF) cpm/5000 cells	MF - mb cpm/5000 cells	Equivalent wt. of $^{45}Ca \times 10^{-12}$ gms in 5000 cells	Equivalent wt. of $^{45}Ca \times 10^{-12}$ gms in 1 cell
30 S.E. $\pm$ 1.44	1063 S.E. $\pm$ 3.98	1033 cpm	84.6×10^{-12} gms	0.017×10^{-12} gms

Mean algal intake of 10 rotifers aged 4 days equals 1500 cells per day corresponding to 25.2×10^{-12} gms of 45 calcium per day (308 cpm).

Algal 45 calcium intake as a percentage of total 45 calcium count in 0.02 mls Control 45 Calcium medium = 0.98%

This count represented less than 1% of the total count obtained for 0.02 mls of unsupplemented Knop's Control 45 calcium medium.

The algal intake of a single rotifer was approximately 1500 cells a day, corresponding to a weight of 25.2×10^{-12} gms of 45 calcium. It should be stressed that this figure was based on only a single day's observation in 4 day old rotifers, and as such was not an accurate quantitative assessment for rotifers of all ages during the life period.

PRELIMINARY DISCUSSION

ALGAL CALCIUM UPTAKE EXPERIMENT

The results of the foregoing experiment indicate that comparatively little change in the overall specific activity of culture droplets takes place as a result of ingestion of 45 calcium by the algal component of cultures, 5000 algal cells took up less than 1% of the total 45 calcium count obtained for a 0.02 ml droplet of Knop's Control 45 calcium medium. However, on the basis of a very approximate estimate of the daily algal intake for a 4 day old rotifer, a potential daily uptake of 45 calcium corresponding to 308 cpm (25.2×10^{-12} gms) could occur. Undoubtedly, much of the algal intake is excreted, and this point is further discussed in the following discussion in relation to the 45 calcium accumulated in early life in rotifers continuously exposed to this radionuclide in Knop's Control 45 calcium medium.

DISCUSSION - PART 2

Antioxidants have been classified by Passwater 1971 into four categories, Mis-synthesis resorters, i.e. seleno-amino acids and selenium, Membrane stabilizers, i.e. the naturally occurring antioxidant Vitamin E., Activators, i.e. iron, copper and Vitamin C, and Free-radical scavengers, i.e. B.H.T., ethoxyquin etc. The three

antioxidants employed in this present study belong to the category of free radical scavengers, although it should be noted that one of them cysteine hydrochloride is a specific protector against the results of exogenous ionising radiation.

The water soluble anti-oxidants, B.H.T., sodium hypophosphite and cysteine hydrochloride were added as a constant supplement (0.01%) of the Knop's Control culture medium, consequently rotifers were exposed to a particular antioxidant throughout life and not as a brief treatment on alternate days, see Chelation procedures Page 48 The greatest increase in life expectancy of 52% over the untreated Controls was shown by the B.H.T. treated group, while increased life expectancies of 37.6% and 22.3% were shown by the sodium hypophosphite and cysteine hydrochloride treated groups respectively. Harman in 1957 reported a 6.7 X survival increase in populations of mice that had received 0.5% weight of B.H.T. antioxidant in their diet, and in a paper earlier in the same year the same author reported a 20% increase in half survival time of Akr mice treated by a 1% supplement of cysteine hydrochloride in their diet. In his most recent paper Harman 1969 reported mean life periods of 17.5 $\pm$ 5 months and 20.9 $\pm$ 4.7 months in two populations of mice maintained on diets containing 0.25% and 1% of B.H.T. by weight, respectively. The untreated Controls exhibited a shorter life period of 14.5 months. In his 1969 study Harman adopted a semi synthetic base diet containing 20% by weight of casein, and he emphasises that although the B.H.T. treated groups demonstrated significant increases in life expectancy their life-spans were still significantly less than those for the Control mice receiving an optimum commercial diet. A much propounded criticism of the studies performed on mice and rats that demonstrated increased life expectancy as a result of treatment with antioxidants added to synthetic diets has been the failure of such studies to reveal similar increases in life expectancy in the same populations maintained on optimal commercial diets. Indeed, it has even been suggested that a synthetic diet supplemented with antioxidant was less palatable than its unsupplemented equivalent,

hence increased life expectancy occurred as the result of dietary restriction. In the case of the antioxidant treated rotifer populations a non synthetic dietary regime was employed, and no evidence was obtained that indicated this regime deviated from the optimum for the species under laboratory conditions.

It has been noted previously that the level of egg-laying has often been used in ageing studies to reflect the level of metabolism (Lansing 1942) and that such studies sought to investigate the effects of ageing without also seriously disrupting the level of metabolism in the particular organisms concerned (see Page 12). In this study only one population, the cysteine hydrochloride treated group, exhibited a reduced egg-laying rate compared to the untreated Controls. That this group also showed a 22.3% increase in life expectancy may thus have been attributable to a reduced metabolic status and not necessarily a delay in the ageing process per se. Alternatively, the ageing process may have been delayed, the antioxidant medium simply deviating from the optimum for some individuals in respect of the rate of egg-laying. In the specific context of egg-laying it is also noteworthy that both the B.H.T. and cysteine hydrochloride treated groups showed a delay in the onset of egg-laying, particularly in the case of the B.H.T. group which showed a high rate of egg-laying relative to the untreated Controls during its reproductive period. Lansing in 1954 has described delay in the onset of egg-laying as being associated with pediaclones, orthoclones whose life spans showed increases over generations of orthoclone selection, and this is particularly interesting in relation to the cysteine hydrochloride and B.H.T. groups which showed increased life expectancy, particularly in the case of the B.H.T. treated population where the greatest increase in life expectancy recorded for any of the experimental groups was also associated with the greatest delay in the onset of egg-laying.

The results of the population cultured on Knop's Control radioactive culture medium, supplemented to a total salt concentration of 0.05% with the antioxidant B.H.T., showed a non uniform and sporadic accumulation of 45 calcium that was at all times much slower than the uniform accumulation of 45 calcium in the untreated 45 calcium Control group. This was a particularly interesting result as it links the age-retarding effects of antioxidation with the specific accumulation pattern of an ion implicated as an important factor in the ageing process of rotifers.

A reduction in dietary level from 5000 cells per 0.02 mls of culture medium to 1900 cells per 0.02 mls resulted in increased longevity in all 3 experimental populations cultured on the 3 modified Knop's media described in the Ion Ratio Experiments, without accompanying reductions in fecundity or delayed growth. It should be mentioned that growth was assessed at its conclusion only on the basis of size measurements and that although no reduction in fecundity was evident in the nutritionally restricted groups, two of them, the restricted Control and Low calcium groups, showed a delay in the onset of egg-laying.

The studies performed by McCay et al in 1934 and 1935 employed synthetic nutritionally restricted regimes that differed from the full synthetic diet only in relation to total calorific value. The white rats maintained on the restricted diets showed increased life expectancy compared to the Control groups of the relevant sex grown on the full diet. In all cases increased longevity was associated with delayed growth, growth being assessed in terms of total body weight, and delayed maturation. No delay in growth (assessed in terms of size) was noted in the nutritionally restricted groups that showed increased longevity in this study, and although a slight delay in onset of egg-laying occurred in two groups, it did not indicate more than a slight delay in the attainment of reproductive maturity. It is worth mentioning that in the nutritional study of Fanestil and

Barrows 1965 (described in the INTRODUCTION, DIETARY RESTRICTION EXPERIMENTS) rotifers of the species Philodina acuticornis cultured on their diet 3 (the lowest nutritional regime), also showed a slight delay in the onset of egg-laying, and although the length of the reproductive period was extended into the prolonged parts of the life period, the levels of egg-laying were greatly reduced in comparison with the fully fed Controls over the entire reproductive period. This was not the case in the nutritionally prolonged groups in the present study. No information on the growth rate in the nutritionally restricted groups was provided in the study of Fanestil and Barrows, however assays of malic and lactic dehydrogenase activities in the dietary restricted and fully fed groups indicated a delay in the age related decrements in the activities of these two enzymes in the former populations.

The measurements carried out on the 45 calcium potentially taken up by rotifers in the algal diet ingested indicate that a significant accumulation of 45 calcium could be derived from this source, corresponding to approximately 308 cpm per day (i.e. the maximum rate of uptake of calcium observed in Radiotracer Experiment 1). No information is available on the fraction of the dietary intake that is excreted on each day of life, however assuming the number of algal cells ingested by rotifers during the period of growth was of the order of 1500 cells per day, it is quite apparent from the results of continuous 45 calcium exposure experiments in Radiotracer Experiment 1 that all the 45 calcium potentially accumulated from the dietary intake of algae, was actively exchanged back to the culture medium by excretion during growth. However, the algal intake following the growth period may have contributed significantly to the total calcium accumulated during later life, when the accumulation of calcium could be due to a failure in the excretory mechanism. It may be postulated that in the absence of the sort of delayed growth and maturation previously noted by McCay in animals maintained on reduced diets, the reduced calcium intake by way of algal ingestion, at the lower nutritional level of 1900

cells per 0.02 mls, may well have been a contributory factor in the resultant increase in life expectancy.

The antioxidation and dietary restriction experiments undertaken in this study constitute only the preliminary steps to further experiments. While only tentative arguments may be put forward at this stage, it is suggested that neither of the results from these approaches are in conflict with a thoery of ageing in rotifers that involves the detrimental accumulation of an ion species during the life period.

TABLE 19

key * = DELAY IN ONSET OF REPRODUCTIVE PERIOD

CULTURE MEDIUM	TREATMENT GIVEN	MEAN LONGEVITY	INCREASE IN LONGEVITY	TOTAL EGGS LAID	REPRODUCTIVE PERIOD	NO. OF SURVIVORS COUNTS PER MINUTE ETC.	DAYS OF LIFE 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
CONTROL		8.7 ± 0.2 days		136	7 days	No. of Survivors	30	30	30	30	29	29	26	20	7										
						Counts per Minute			56	156	272	330	539	774	1060										
						Rate of food intake per day				1500 cells															
						45 Ca in daily food intake				308 cpm															
HIGH Ca		7.6 ± 0.2 days	- 12.6%	98	7 days	No. of Survivors	30	30	30	27	27	23	18	10	4										
						Counts per minute			132	248	349	466	650	1043											
LOW Ca		12.2 ± 0.3 days	40.2%	210	10 days *	No. of Survivors	30	30	30	30	29	29	29	28	26	23	21	15	9	5					
						Counts per minute			32	57	69	83	112	153	218	318	666	780	814						
CONTROL	DIET RESTRICTION	11.8 ± 0.2 days	34.1%	242	10 days *	No. of Survivors	30	30	30	30	30	30	30	30	25	21	17	12	8	2					
HIGH Ca	"	10.9 ± 0.3 days	23.8%	212	10 days	No. of Survivors	30	30	30	30	29	28	24	24	24	20	20	8							
LOW Ca	"	15.8 ± 0.3 days	79.5%	368	13 days *	No. of Survivors	30	30	30	30	30	30	30	30	30	28	28	26	23	20	17	12	8	2	
LOW TO CONTROL		9.1 ± 0.2 days		146	6 days *	No. of Survivors	30	30	30	30	30	27	26	15	8	6									
HIGH TO CONTROL		8.0 ± 0.3 days		106	6 days	No. of Survivors	30	30	29	29	29	28	18	8	6	4									
LOW TO HIGH		8.6 ± 0.2 days		100	6 days *	No. of Survivors	30	30	30	30	29	29	25	11	9	4									
CONTROL TO HIGH		7.9 ± 0.2 days		103	7 days	No. of Survivors	30	30	30	30	26	26	20	10	4										
CONTROL TO LOW		12.3 ± 0.3 days		239	10 days	No. of Survivors	30	30	30	30	30	30	28	28	26	23	20	17	12	6					
HIGH TO LOW		12.0 ± 0.3 days		209	10 days	No. of Survivors	30	30	30	30	29	29	26	26	25	25	21	16	10	3	1				
CONTROL	0.5% Na CITRATE	13.2 ± 0.3 days	51.7%	223	12 days *	No. of Survivors	30	30	30	30	29	29	29	28	26	26	23	20	16	11	5	3			
						C.P.M. in ROTIFERS					60		100		198		300		508		782				
						C.P.M. in WASHINGS			57		94		128		140		200		315		405				
						%age Ca Withdrawn			100		61		56		41		40		38		34				
CONTROL	0.25% EGTA	15.3 ± 0.2 days	75.9%	243	12 days *	No. of Survivors	30	30	30	30	30	30	30	30	30	30	28	28	25	22	14	3			
CONTROL	0.25% EDTA	13.0 ± 0.3 days	49.4%	202	11 days *	No. of Survivors	30	30	30	30	30	30	30	30	30	27	23	18	13	6	3				
CONTROL	0.25% Na TARTRATE	12.5 ± 0.2 days	43.7%	190	10 days *	No. of Survivors	30	30	30	30	30	30	30	30	30	26	20	14	9	6	1				
CONTROL + Antioxidant	B.H.T.	12.9 ± 0.2 days	52.0%	284	11 days *	No. of Survivors	30	30	30	30	30	30	30	30	30	27	26	15	9	6	3				
						Counts per minute			50	65	60	70	80	165	113	235	120	120	115	240	300				
CONTROL + Antioxidant	Na HYPOPHOSPHITE	11.7 ± 0.2 days	37.6%	253	10 days	No. of Survivors	30	30	30	30	30	30	30	29	26	22	15	11	4	3	1				
CONTROL + Antioxidant	CYSTEINE HYDROCHLORIDE	10.4 ± 0.3 days	22.3%	110	10 days *	No. of Survivors	30	30	30	30	30	30	27	25	17	14	6	3							

SUMMARY (See Table 19)

Rotifers of the species Mytilina brevispina var redunca were cultured at 24°C under standard and aseptic culture conditions in artificial saline media of three calcium concentrations.

1. All populations were homologated with respect to maternal age by a process of egg selection carried out over three generations.

2. Observations on survival, growth and egg production were made on populations cultured on the three media containing a full and reduced diet, while the effects of transfer between culture media, periodic washing in chelating agents, and constant exposure to antioxidants were tested on survival and egg-laying in Control cultures.

3. The calcium content of rotifers was investigated by continual exposure to the radionuclide 45 calcium in the three culture media, while the 45 calcium accumulated and withdrawn from rotifers cultured on Control 45 calcium medium and subjected to treatment with the chelating agent sodium citrate, and the 45 calcium accumulated in rotifers cultured on Control 45 calcium medium containing the antioxidant B.H.T., were also investigated. The 45 calcium intake of algae cultured for one day on Control 45 calcium medium at the concentration normally employed for experiments was noted.

4. In the case of rotifers cultured on a full diet in the three calcium media the greatest longevity, egg total, and reproductive period was recorded in the Low calcium cultured population, while the lowest longevity value and total number of eggs laid was recorded in the High calcium group. Continual exposure to 45 calcium in each of the three media, revealed an accumulation of calcium that began

in all cases at the end of the period of growth in size. This accumulation occurred at a rate that was inversely related to longevity and directly related to the level of radionuclide in each medium in the manner predicted by the Lansing hypothesis. The total 45 calcium taken up in the daily intake of algae in 4 day old rotifers cultured on Control medium, was of approximately the same order as the maximum rate of 45 calcium accumulation in untreated rotifers cultured on the same medium, suggesting a possible source of calcium accumulation in the event of a breakdown in the mechanism of excretion.

5. Rotifers cultured on a reduced diet in each of the three media showed an increase in longevity, egg total and reproductive period compared with rotifers cultured in the corresponding media on a full diet. However, the relative differences with respect to these characteristics were approximately similar between populations cultured on the same dietary level, with the Low calcium cultured population showing the greatest increase in longevity, total egg production and reproductive period. No extention in the growth period or difference in final size was noted in the case of the dietary restricted groups (cf McCay's starvation studies).

6. The results of the culture transfer experiments in which rotifers were transferred to another of the three media at the end of the growth period, revealed that it was the medium on which rotifers were cultured after growth that exerted the major influence on longevity value, total egg production and length of reproductive period. This result is in agreement with the appearance of an ageing factor at the end of the period of growth in size postulated in the Lansing ageing theory.

7. Rotifers cultured in Knop's Control medium and subjected to periodic, brief immersions in chelating agents showed increases in their longevity value, total egg production and reproductive period. There was evidence that these increases were directly related to the relative specificity of the chelating agent for calcium (measured in terms of the log b value of formation constant at a given temperature and ionic strength). The 45 calcium accumulated in rotifers subjected to periodic immersion in the chelating agent sodium citrate increased at a lower rate than that of the untreated Controls, while the 45 calcium present in sodium citrate washings increased at each successive treatment throughout life. The sum of the calcium accumulated and withdrawn for citrate treated rotifers did not equal the level of 45 calcium accumulation of the untreated Control group on any day of life after day 3, and this could indicate that the citrate treated rotifers quite apart from showing a reduction in the overall level of 45 calcium accumulated, also showed a lower rate of 45 calcium intake between treatments. The percentage calcium withdrawn as a fraction of the total calcium present before treatment decreased throughout life, with the greatest reduction occurring at day 5, when it may be postulated a marked increase in the inexchangeable fraction of calcium accumulated took place.

8. Rotifers cultured on Knop's Control media supplemented with antioxidants showed increases in longevity value, length of reproductive period and with the exception of the cysteine hydrochloride treated group - total egg production. Rotifers cultured on Control 45 calcium medium supplemented with the same concentration of the antioxidant B.H.T. used previously, showed a markedly reduced calcium uptake compared with the untreated Controls.

9. All populations that displayed increased longevity with the exception of the sodium hypophosphite treated group, also showed a delay in the onset of the reproductive period. This delay was not associated with an extended growth period in the case of populations reared at the three calcium concentrations on full and reduced diets, or rotifers cultured at the Control concentration of calcium and subjected to treatments with sodium citrate.

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Preliminary Studies of the In Vitro Cellular Effects of Asbestos and Fine Glass Dusts

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Considerable evidence now supports the fact that asbestos dusts induce tumors in vivo (Wagner 1960; Selikoff et al. 1965; Wagner et al. 1973). However, little evidence exists relating to the mechanistic activity of asbestos at the cellular level. The present study was undertaken to investigate the cellular effects of asbestos and, in particular, the possibility that asbestos and fine glass dusts could induce karyotypic changes when introduced into cell-culture regimes.

A series of preliminary experiments were undertaken to ascertain whether or not asbestos could induce morphological transformation in vitro in the case of a contact-inhibiting cell line, murine 3T3 (Flow Laboratories, Irvine, Scotland). The asbestos samples used were S. F. A. chrysotile and U. I. C. C. crocidolite. They had been autoclaved before being suspended in phosphate-buffered saline (Dulbecco, modification A) and added at a final concentration of 0.01 mg/ml, with accompanying gentle agitation, to 75-cm^2 glass bottles containing 15 ml Dulbecco-Vogt modified Eagle's medium (15% fetal calf serum) in which 1.7×10^5 3T3 cells were suspended. Similar small aliquots of saline were added to the nonfiber control flasks. Two replicate 75-cm^2 bottles were selected for each test sample, which also included a coarse fiberglass control selected because of its inability to produce tumors in vivo (Wagner et al. 1973). All flasks were observed daily for at least 10 days.

At 7 days, areas of multilayered growth were observed in the asbestos-treated cultures (see Table 1 and Fig. 1). These layers consisted of small foci of densely packed cells which rapidly increased in area during the subsequent days of observation. Additional foci were observed after 7 days, but it was difficult to assess whether or not these represented freshly instigated multilayers or derivatives of the original focal sites. Subsequent subculture of the asbestos-treated flasks led to the formation of more focal multilayers which, after approximately three passages, had completely outgrown the contact-inhibiting 3T3 cells. No multilayered growth was observed in the coarse fiberglass or unexposed control cultures during this period.

Table 1
Effects of Asbestos Treatment on 3T3 Cells

	Days of culture									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
S. F. A. chrysotile							3, 2[a]	5, 4	7, 6	9, 11
U. I. C. C. crocidolite							1, 3	2, 4	5, 7	8, 7
Coarse fiberglass										
Control										

[a] The two numbers indicate the numbers of foci in replicates.

Cultured explants of the focal areas together with the non–contact-inhibiting asbestos-treated cultures were treated with 0.04% colchicine for 4 hours before being fixed and stained according to the trypsin-Leishman banding procedure of Seabright (1971). Although the resulting karyotypes showed some

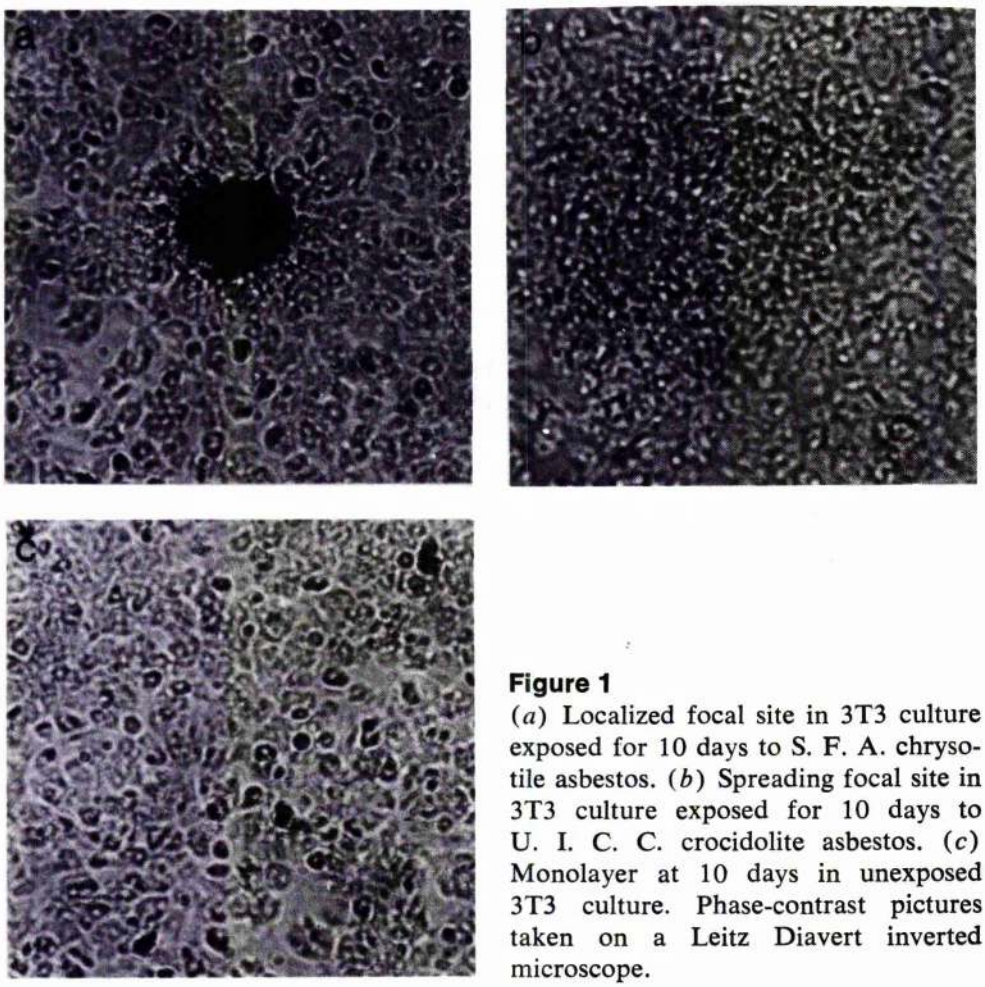


Figure 1
(*a*) Localized focal site in 3T3 culture exposed for 10 days to S. F. A. chrysotile asbestos. (*b*) Spreading focal site in 3T3 culture exposed for 10 days to U. I. C. C. crocidolite asbestos. (*c*) Monolayer at 10 days in unexposed 3T3 culture. Phase-contrast pictures taken on a Leitz Diavert inverted microscope.

genetic changes that appeared restricted to the asbestos-treated cells, they proved difficult to quantify because of the high modal variability of this cell line. As a result, an alternative cell line, CHO-K1 (American Type Culture Collection) (Fig. 2), which displayed a well-marked karyotype of low modal number (hypodiploid stem-line number = 20) as well as a low modal variation of 23%, was adopted for subsequent genetic studies when careful cloning programs had reduced modal variability to as little as 6%.

For genetic studies, cells which had been cloned from a single stem-line cell and checked for a total modal variation of 6% or below were seeded at a density of 2×10^4/ml into 25-cm^2 plastic Falcon containers to which had been added 5 ml of McCoy's 5a medium (Iwakata's and Grace's modification) enriched with 15% fetal calf serum. All dust samples were sterilized and added to the flasks in the manner previously described to give a final dust concentration of 0.01 mg/ml. The control flasks without dust also received a similar small volume of phosphate-buffered saline in which dusts were dis-

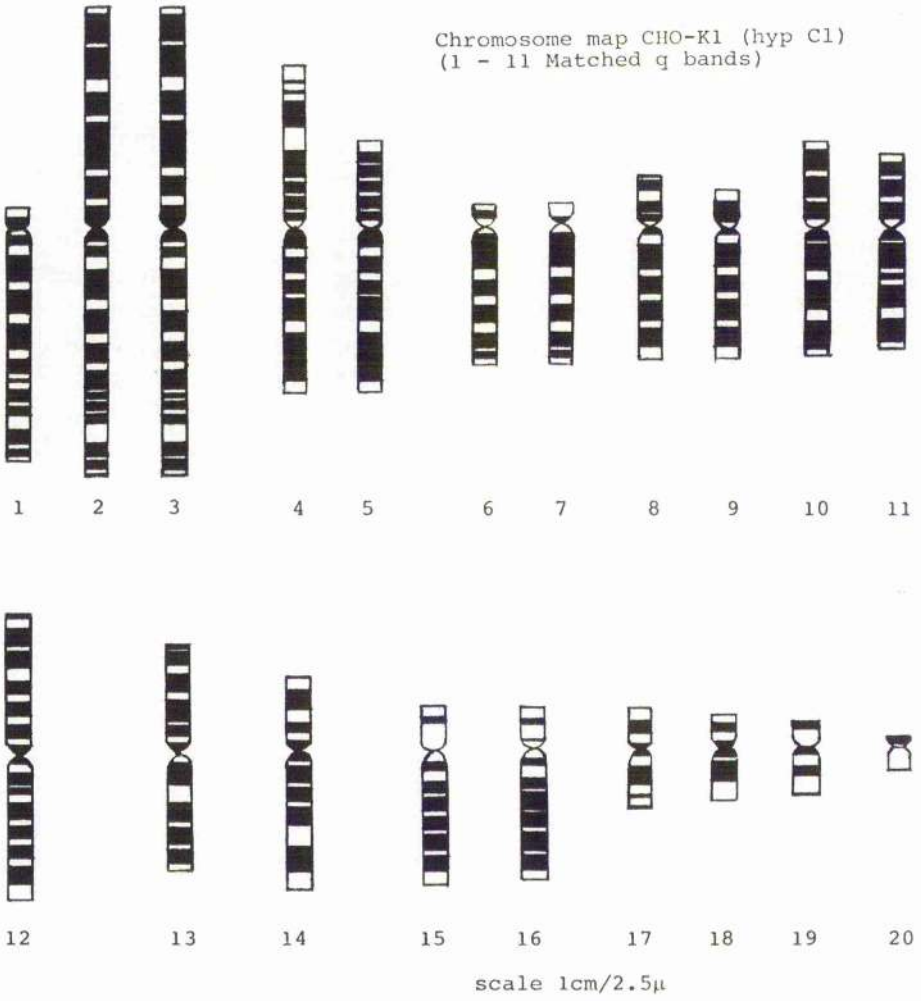


Figure 2
Schematized chromosome map of a hypodiploid CHO-K1 clone (1–11 matched q bands). Scale: 1 cm/2.5 μm.

pensed. The asbestos samples included S. F. A. chrysotile, Rhodesian chrysotile a, Canadian chrysotile b, U. I. C. C. crocidolite, U. I. C. C. amosite, and U. I. C. C. anthophyllite. A coarse fiberglass was again included within the experiment as a fiber control. Each sample, coded to permit statistically blind analysis of the final karyotypes, was added to two replicate flasks containing cells in suspension. After 48 hours of incubation at 37°C, each flask was treated with 0.04% colchicine for 3½ hours before its contents were harvested by trypsinization and slide preparations made according to the trypsin-Leishman banding procedure of Seabright (1971). One-hundred karyotypes from each of the experimental samples were then analyzed carefully. It should be pointed out that in preparing the karotypes of dust-exposed cells it was not considered necessary to adopt in situ methods of fixation since it had already been shown (Sincock and Seabright 1975) that there is no significant difference between asbestos-treated karyotypes subjected to either normal or in situ protocols.

The results of genetic analysis after sample decoding are shown in Table 2, from which it is apparent that, in the case of two of the asbestos samples, S. F. A. chrysotile and U. I. C. C. crocidolite, a high level of aberration had taken place after 48 hours, i.e., more than 50% of the cells analyzed were karyotypically abnormal. The other asbestos samples also showed significant levels of genetic abnormality; however, by way of contrast, no significant genetic damage was observed in the coarse fiberglass or untreated control cultures. It is interesting to note with respect to the arbitrarily chosen categories of abnormality that, with the exception of amosite and anthophyllite, all asbestos samples gave rise to high levels of polyploids comprising in the main cells with twice the modal number of chromosomes. Anthophyllite stands out particularly sharply on the basis of the low polyploid levels scored (2% as compared to the 28% polyploid fraction attributed to S. F. A. chrysotile). All the asbestos samples produced similar numbers of cells with fragments; however, much variation was observed with respect to the category "other abnormalities," which included inversions, gaps, rings, and sister-chromatid exchanges. S. F. A. chrysotile and U. I. C. C. crocidolite produced high levels of aberration within this category, and some of the karyotypes analyzed are shown in Figures 3 and 4. It should be stressed that there was a considerable range of genetic damage observed within the asbestos-exposed cultures, varying from gross aberration (Fig. 4a) involving chromatic pulverization with accompanying presence of many fragments to more minor damage involving only a few genetic lesions (Fig. 4c,e,f). Both asymmetrical and symmetrical types of genetic aberrations were observed in all asbestos cultures, and Figure 4d depicts asymmetrical and symmetrical changes within a single cell.

To test whether or not a fine-glass-fiber sample, code 100, containing fibers within the diameter and length ranges of asbestos could also produce genotypic abnormalities under similar experimental conditions, the experiment was repeated using the following glass dusts: fine glass, code 100; coarse fiberglass; and glass powder (a coarse borosilicate). In addition, glass samples of uniform 2-μm diameter cut on a Cambridge microtome to lengths of less than 10 μm, 25 μm, 50 μm, and 100 μm were also included in order to ascertain whether by varying the length at a fixed low diameter variations would also be

Table 2
Effects of Different Treatments on Chromosomes

	S. F. A. chryso-tile	*Rhodesian chryso-tile a*	*Canadian chryso-tile b*	*U. I. C. C. crocido-lite*	*U. I. C. C. antho-phyllite*	*U. I. C. C. amosite*	*Glass 110*	*Control*
Polyploids	28	23	27	26	2	14	3	4
Cells with fragments	13	14	11	10	10	16	0	0
Other abnormalities	33	9	15	29	9	13	0	0
Percent abnormal karyotypes	62	34	39	56	26	41	3	4

Results were obtained using 48-hr exposure; 100 cells were analyzed from each culture. Categories of genetic damage were not mutually exclusive.

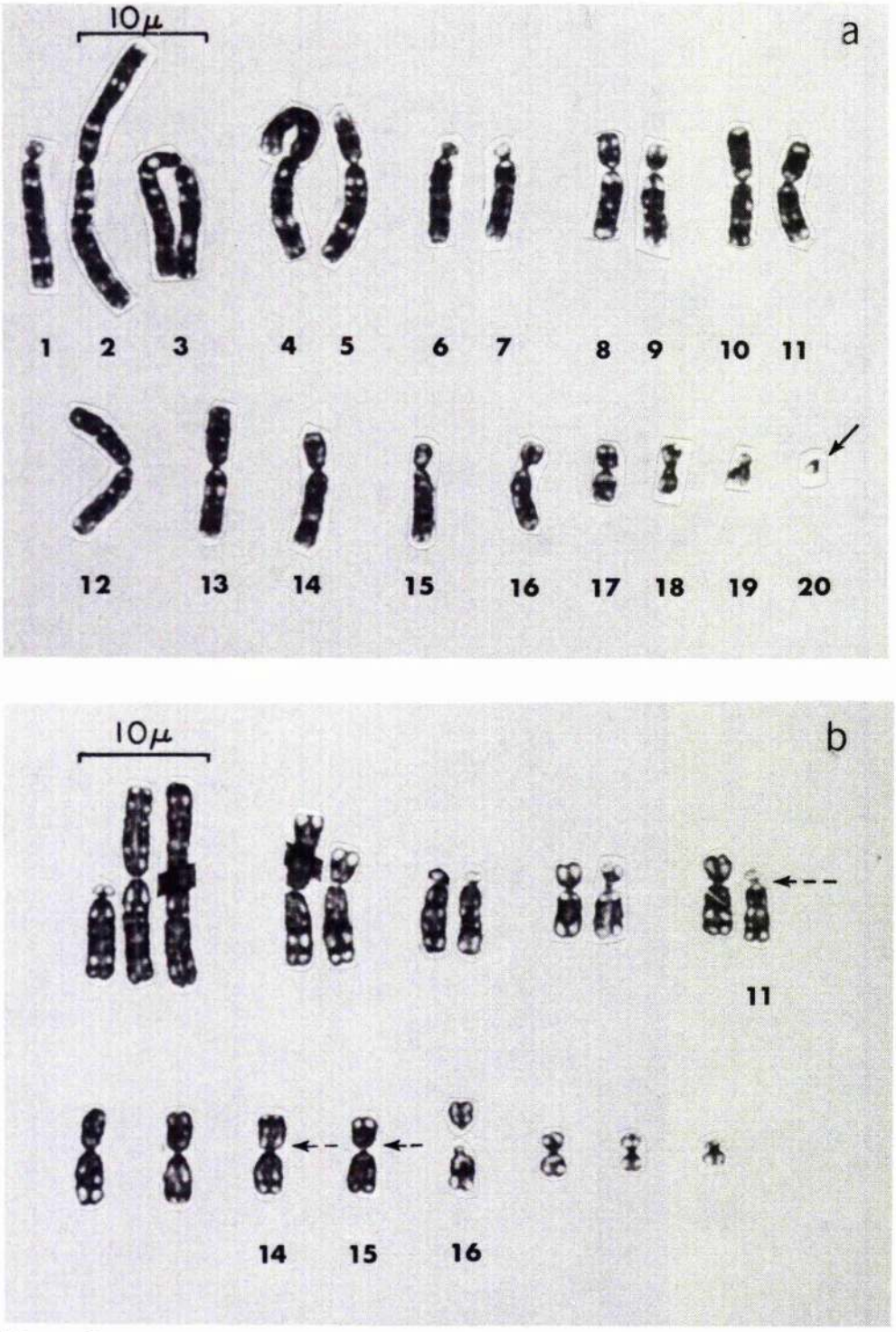
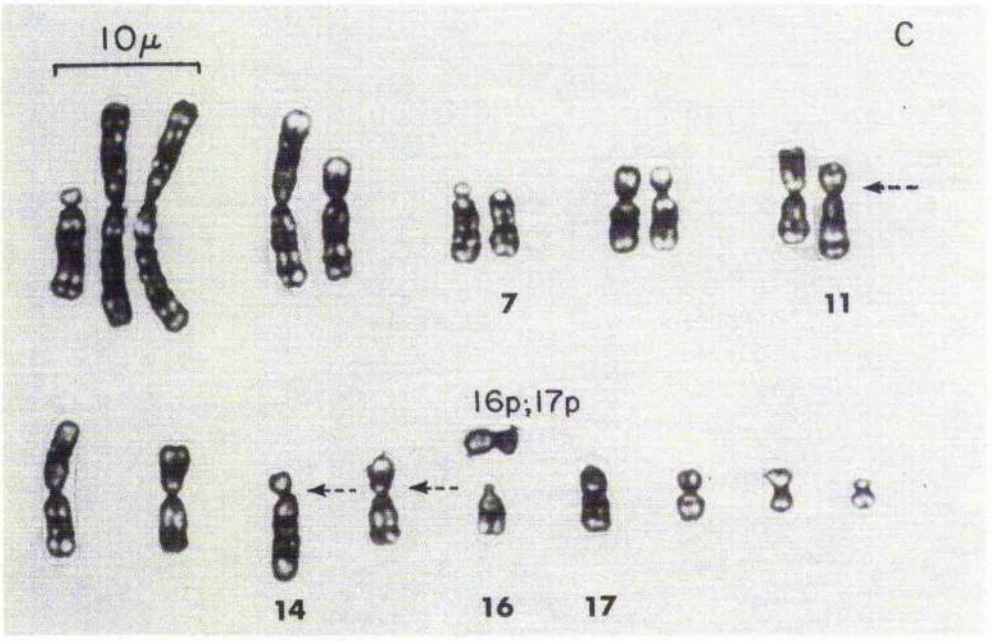


Figure 3

(*a*) Karyotype of unexposed CHO-K1 cell. (Reprinted, with permission, from Sincock and Seabright 1975.) (*b*) Karyotype of a cell exposed to U. I. C. C. crocidolite for 48 hr. Dashed arrows indicate pericentric inversions. Chromosome 16 has broken in the region of its centromere. Marker chromosome 20 is absent.



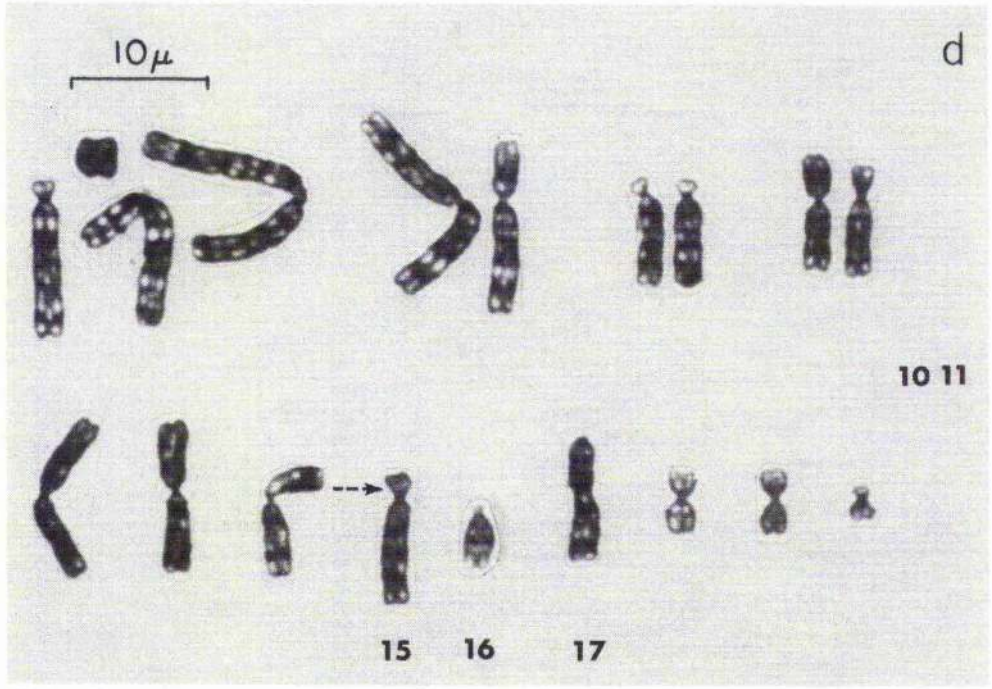


Figure 3 (*continued*)
(*c*) Karyotype of a cell exposed to S. F. A. chrysotile for 48 hr. Dashed arrows indicate pericentric inversions. The short arm of chromosome 7 is missing and the short arms of 16 and 17 are associated in a translocation figure. (*d*) Karyotype of cell exposed to Canadian chrysotile b for 48 hr. Dashed arrow indicates pericentric inversion. Numbers 10 and 11 are missing, and 16 has broken in the region of its centromere. Number 17 has undergone complex rearrangement and a ring is present.

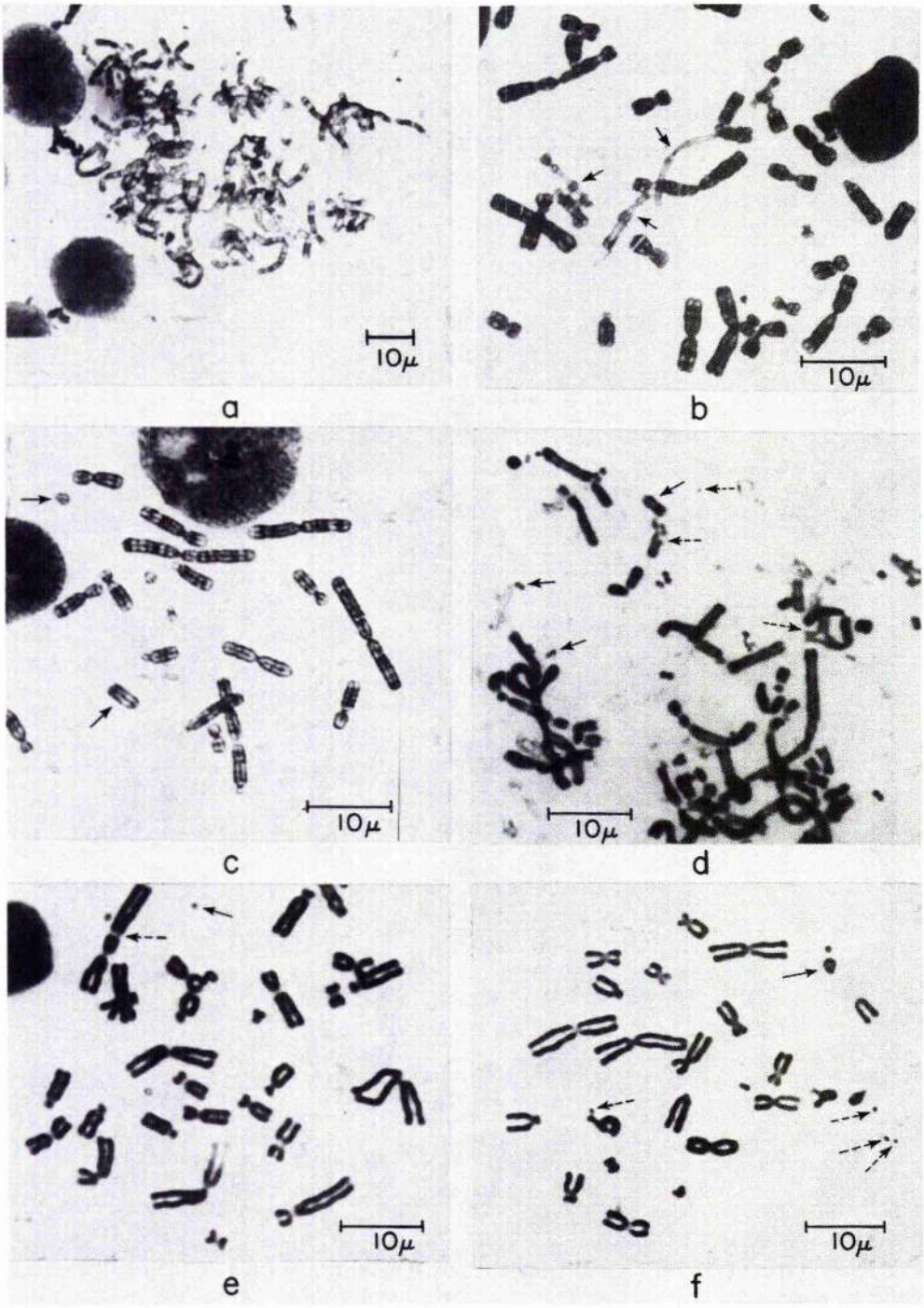


Figure 4

(*a*) Polyploid cell with multiple fragments. (*b*) Arrows point to beaded chromosomes. (*c*) Arrows point to chromosomes with breaks. (*d*) Solid arrows point to symmetrical changes, dashed arrows to asymmetrical changes. (Reprinted, with permission, from Sincock and Seabright 1975.) (*e*) Dashed arrow indicates a chromosome gap, solid arrow a fragment. (*f*) Solid arrow points to ring, dashed arrows to fragments. All the cells shown had been exposed to asbestos.

apparent in the levels of any genetic changes induced. Cells were again exposed to dust samples at a concentration of 0.01 mg/ml for a period of 48 hours.

The resulting karyotypes analyzed (100 per sample) revealed, after sample decoding, a significant level of aberration in the cells treated with glass, code 100, but no significant damage in the case of the other experimental cultures. Compared with the previous asbestos-dust results, the cultures treated with glass, code 100 (see Table 3), showed a level of polyploidy that correlated more closely with the levels produced by amosite and anthophyllite (Table 2) than with the higher polyploid levels found in the other asbestos cultures. Although a similar number of cells with fragments were still scored, with the possible exception of the amosite sample, the number of other abnormalities in the cultures treated with glass, code 100, was well below that found in the cells treated with S. F. A. chrysotile and U. I. C. C. crocidolite but of the order of the values attributed to the other asbestos samples.

Finally, an experiment was undertaken to investigate the effects of chemical leaching and mechanical milling of asbestos fibers on their ability to induce genetic damage in vitro. The same experimental protocols were adopted as before; however, the samples used consisted of Rhodesian chrysotile a, Rhodesian chrysotile a (chemically leached), Canadian chrysotile b, Canadian chrysotile b (chemically leached), U. I. C. C. crocidolite, U. I. C. C. crocidolite (mechanically milled), and coarse fiberglass (fiber control). The fiber-leaching method involved controlled acid extraction followed by washing to neutral pH, and the milling involved a 16-hour mechanical disruption at the mill with concomitant loss of much fiber morphology. The fiber samples were again applied at concentrations of 0.01 mg/ml for a period of 48 hours.

The results of the genetic analysis of 100 cells from each culture, after decoding (Table 4), indicated that both chemical leaching and mechanical disruption exerted a significant reducing effect on the capacity of asbestos to induce in vitro genetic damage. Indeed, the leached Rhodesian chrysotile-a sample produced no significant genetic damage at all, and both the Canadian chrysotile-b and the mechanically pulverized U. I. C. C. crocidolite samples showed only small increases in genetic abnormalities over the experimental controls.

The results of the previous studies indicate that both positive transformation of morphology and positive genetic responses may be achieved by the passive inclusion of asbestos in cell-culture regimes. The formation of transformed cell foci after only 7 days and the rapid growth rate of the cells comprising the focal sites (only three passages being required for them to outgrow their contact-inhibiting peers) demonstrate the rapidity and extent to which this response may be achieved. Similarly, the genetic effects produced in Chinese hamster cells after only 48 hours of exposure to asbestos dust emphasize this same point. The facts that a fine glass, code 100, also produces cellular genetic damage and that loss of physical structure by milling also results in a reduction of this effect could indicate the importance of physical morphology in these responses. However, chemically leached fibers also show a reduction in their chromosome-damaging effect, and this could indicate either that there are chemical inclusions in the fibers that are implicated in this genetic activity, or that leaching produces damage to the

Table 3
Effects of Different Treatments on Chromosomes

	Glass 100	*Glass 110*	*Glass powder*	*Glass[a] <10 μm long*	*Glass[a] 25 μm long*	*Glass[a] 50 μm long*	*Glass[a] 100 μm long*	*Control*
Polyploids	8	6	4	8	5	7	4	5
Cells with fragments	10	0	0	0	0	0	0	0
Other abnormalities	12	0	0	0	0	0	0	0
Percent abnormal cells	23	6	4	8	5	7	4	5

Results were obtained using 48-hr exposure; 100 cells were scored from each sample. Categories of genetic damage were not mutually exclusive.

[a] Glass samples of uniform 2-μm diameter.

Table 4
Effects of Different Treatments on Chromosomes

	Rhodesian chryso-tile a	*Rhodesian chryso-tile a leached*	*Canadian chryso-tile b*	*Canadian chryso-tile b leached*	*U. I. C. C. crocido-lite*	*U. I. C. C. crocido-lite milled*	*Glass 110*	*Control*
Polyploids	23	6	26	10	26	6	6	4
Cells with fragments	13	0	9	0	14	9	0	0
Other abnormalities	10	0	16	4	28	3	0	0
Percent abnormal cells	34	6	42	14	57	16	6	4

Results were obtained using 48-hr exposure; 100 cells were scored from each culture. Categories of genetic damage were not mutually exclusive.

Table 5
Effects of Different Treatments on Chromosomes

	U. I. C. C. crocido-lite	*U. I. C. C. crocido-lite*[a]	*S. F. A. chryso-tile*	*S. F. A. chryso-tile*[a]	*Controls*
Polyploids	22	6	24	18	3
Cells with fragments	15	0	16	0	0
Other abnormalities	25	8	28	8	0
Total percent abnormal cells	51	14	59	20	3

Results were obtained using 48-hr exposure; 100 cells were scored from each sample. Categories of genetic damage were not mutually exclusive.
[a] Preincubated 48 hr in medium.

physical structure of fibers, thus rendering them inactive as a genetically damaging species.

It is important not to overlook the facts that many complex interactions occur between asbestos dusts and the media in which cells are cultured, and that these may have an influence on the overall in vitro effects produced. It can be shown that if asbestos fiber is preincubated for 48 hours before it is applied to cell cultures, then the positive genetic effects which it induces are severely reduced (see Table 5). It is known that asbestos can be extremely porous (Gorski et al. 1976), and the possibility that such components as serum proteins may be readily absorbed onto the fiber surface, thereby forming a coating on the active structure, cannot be ruled out. This type of structural modification could explain why it has frequently been observed that an increase in the time cells receive exposure to asbestos does not necessarily result in a concomitant increase in the level of genetic damage produced.

The types of genetic damage produced by asbestos and fine glass, code 100, have already been presented under a few arbitrarily chosen headings and have been briefly compared. The illustrations also delineate the sort of damage that is encountered within individual karyotypes. From the karyotypes analyzed, it would be true to say that there is a tremendous range of aberrational effects, including both asymmetrical and symmetrical responses. However, it has been noticed that pericentric inversions and centromeric breaks, the latter often leading to a complex translocation figure, are among the more commonly encountered aberrations when one excludes polyploids and fragments arising from other break points. It is interesting to note that the tiny marker chromosome 20 (see Fig. 3a), which banding has revealed to be a centric chromosome rather than an acentric fragment, has often been found to be absent in abnormal karyotypes irrespective of the level of other abnormalities. For this reason, this particular karyotypic loss, which could be explained in terms of dust-induced anaphase lag involving damage to the spindle mechanism or centromeric attachment, was often adopted in early studies as a marker for aberrant karyotypes in its own right.

The question now arises as to whether the genetic changes reported here are in any way relevant to the carcinogenic activity of asbestos fibers. Certainly, positive genetic responses from a physical fiber of this type could pro-

vide a route toward a stem line of aberrant genotype, and this would be supported by the observation that many of the karyotypes analyzed contained only minor chromatic lesions, which undoubtedly would be heritable over a number of generations. It is certainly true that CHO-K1 cells are extremely labile with respect to the response shown when asbestos is included in their regimen, and this applies equally to the inclusion of pesticides such as Guthion (Alam et al. 1974).

The well-marked karyotype from an established cell line such as CHO-K1 has undergone considerable postcrisis breakdown before reaching a delicate equilibrium at which subsequent breakdown is retarded. In this particular case, this has permitted the stabilization of the karyotype, at least temporarily, at low modal values by careful cloning protocols. The inclusion of any genetically active species will readily upset this fine state of equilibrium and produce a rapid and marked effect, as has been demonstrated. This enlargement of response compared to primary cell responses may well be suited to the quantification of genetic activity of individual types of fibers. Furthermore, the evidence seems to point to the possibility that this system could grade fine dusts on the basis of induced genetic aberration, and if the data so obtained were in any way correlated to respective incidences of tumor production in vivo, they could form part of a composite screening test for the assessment of potential carcinogenic activity of fibrous dusts.

Both the transformation of 3T3 cells and the genetic responses of CHO-K1 karyotypes emphasize the rapidity and extent of the responses that may be achieved by the passive exposure to fine dusts. Furthermore, the production of genetic damage by fine glass, which has been shown to be neoplastic in vivo (Stanton and Wrench 1972), and the reduction of aberration level produced by the breakdown of fiber morphology indicate that the fibrous nature of the dusts plays an important part in the induction of these genetic responses. However, the effects of leaching may in fact result from undetected breakdown of the physical structure of the fibers or they may point toward the chemical composition of fibers as being directly involved in the instigation of this response. It has been pointed out (Roy-Chowdhury et al. 1973) that concentrations of trace metals potentially carcinogenic had been ascribed to samples of amosite, crocidolite, and chrysotile. The negative effects of length variations at a fine diameter of 2 μm may indicate simply that this uniform diameter value lay outside the range for production of genetic response, although glass fibers in the range 0.06–3 μm further milled to approach the lengths of asbestos did have a marked neoplastic effect in vivo (Stanton and Wrench 1972). Bogovski et al. (1973) have implicated fibers less than 2.5 μm in diameter and 10–80 μm long in carcinogenic activity. However, these sizes all refer to nonuniform fiber samples where, unlike in the sample adopted in this study, there may have always been <2-μm fibers present within the variation which produced the carcinogenic activity. Much more variation of diameter would have to be carried out in the case of uniform samples to clarify this point.

Whatever the precise mechanism which produces a positive cell response, it is certainly worthwhile in the case of a carcinogen such as asbestos to proceed toward a greater understanding of its causes insofar as this may enhance knowledge of the cellular activity of the carcinogen in vivo. Finally,

the grading of responses of the type described in CHO-K1 cells and their possible correlation to fiber tumorigenicity make this sort of study a candidate for inclusion in a composite test for the screening of harmful dusts both in industry and in the environment.

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